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THE TECHNIQUE OF PASSIVE HEMAGGLUTINATION AS APPLIED TO STREPTOMYCETES[1]

R. J. DOUGLAS AND E. H. GARRARD

Abstract

A passive hemagglutination technique is described which was used to study the serological reactions of a group of soil streptomycetes. A red blood cell sensitizing material was found in extracts of mycelium disrupted by sonic vibration; acetone-dried powders prepared from these extracts constituted very stable sources of material for serologic tests. Partial purification of the material was achieved by treatment with aqueous phenol and precipitation with ethanol.

Cross reactions observed between different *Streptomyces* species suggested that group antigens were present. Absorption of sera with packed sensitized red blood cells indicated that antigenic material of more restricted distribution also occurred.

Data are presented to indicate that the substances responsible for the passive hemagglutination reaction are present in the cell wall of the mycelium.

Introduction

Currently there are very real problems in microbial classification and these are especially vital with regards to the actinomycetes. In a recent review Waksman (5, 16) has discussed the problem in an attempt to reconcile a scheme of actinomycete classification with the classical taxonomic concepts as applied to higher plants and animals.

The variability of actinomycetes constitutes a problem; much of this variation appears to occur as a response to changes in environmental conditions (16). Other causes of variability have been advanced; Kleineberger-Nobel (8) has suggested that the streptomycetes may alternate between haploid and diploid phases during their life cycle; others have demonstrated that heterokaryon formation may occur (1).

Final definition of a streptomycete as being a strain of *Streptomyces scabies* has often been made on the basis of the pathogenicity of the culture on susceptible tubers (3); this is a questionable criterion, however, considering the existing data on avirulent forms of many pathogenic microorganisms.

Other workers have applied serological procedures to the study of actinomycetes and a variety of modifications of conventional serological methods

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Contribution from the Department of Bacteriology, Ontario Agricultural College, Guelph, Ontario. The investigation was part of the program of the Ontario Potato Scab Research Committee.

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has been devised to overcome the difficulties imposed by the filamentous nature of the organisms. The literature dealing with this work has been discussed by Ludwig and Hutchinson (9) and more extensively by Slack *et al.* (15). Previous work in this laboratory utilized a flocculation reaction (3) but the results were complicated by the occurrence of precipitin reactions of a lower order.

In recent years the technique of passive hemagglutination has become a widely used serological method (11). Basically this technique involves the adsorption of soluble antigen on washed red blood cells, a process which modifies the red cell surface in such a way that the cells become specifically agglutinable by antisera to the adsorbed antigen. This technique has been used with polysaccharide-containing antigen solutions obtained from streptomycin- and luteomycin-producing strains of actinomycetes (17). The present study deals with the application of this method and reports some of the characteristics of the antigen solutions.

The formation by lysozyme of protoplast-like structures from certain *Streptomyces* species has been observed in this laboratory (4). Some serological data arising from these observations are also discussed.

Materials and Methods

The source and identity of the cultures used have been described (4).

For the preparation of immunizing antigens shake cultures were grown at about 27° C in a synthetic medium containing glucose, asparagine, and salts. The mycelium was harvested by filtration, washed and resuspended in 0.85% saline, and disrupted in saline for 5 minutes in a 250-watt 10 kc Raytheon oscillator. The resulting suspensions were injected I.V. into mature rabbits at 3- to 4-day intervals. Most sera had hemagglutinin titers greater than 1:160 after five injections.

Antigen fractions for serum testing were obtained from shake cultures grown in a yeast extract–glucose–salts medium and harvested and washed as above. The mycelium was subjected to sonic disruption in 0.05 *M* phosphate buffer (pH 7.0) and the unbroken filaments removed by centrifugation. Acetone powders were made by the slow addition of sonic extract to 10 volumes of cold stirred acetone. A small amount of 3 *M* acetate buffer (pH 4.9) was added dropwise to promote flocculation. The precipitate was harvested by filtration and washed several times with fresh cold acetone, then dried in an evacuated desiccator over NaOH and anhydrous Na_2CO_3. The dried powders were stored over Drierite at −10° C. Other procedures will be described in the text to follow.

Serum globulins were prepared by ammonium sulphate fractionation (50%) of antisera against *S. scabies* and *S. fradiae*. These were labelled with fluorescein isocyanate by the method described by Goldman and Carver (6). A drop of fluorescein-tagged globulin was added to heat- and alcohol-fixed smears and incubated in a moist chamber for 30 minutes. Smears were then washed in buffered saline, and a cover slip was added. Observations were

made with a Leitz microscope at a magnification of 360 diameters with dark ground illumination. The light source was an Osram HBO 200 mercury arc fitted with blue filters (Zeiss) and an orange filter used in the ocular. Controls consisted of smears unstained or stained with an unrelated fluorescein-tagged immune globulin.

Rabbit red blood cells (r.b.c.) were used in lieu of sheep cells in passive hemagglutination tests in order to avoid any nonspecific agglutination (presumably due to Forssman antigen). Rabbit blood was collected in Alsever's solution, washed three times in saline, and the r.b.c. resuspended to a concentration of 10%. The antigen solution was added to the cells and incubated with frequent mixing at room temperature for 30 minutes. Unadsorbed antigen was removed by washing with saline. For slide agglutination tests a 10% suspension of sensitized r.b.c. was employed; in hemagglutinin titrations 0.25 ml of 1% r.b.c. suspension was mixed with an equal volume of serial twofold dilutions of the serum under test and the tubes incubated at 37° C for 1 hour.

In serum absorptions r.b.c. sensitized with a heterologous antigen, then washed and packed by centrifugation, were used as the absorbing agent.

Results

Preparation of Red Cell Modifying Antigens

Sonic extracts clarified by centrifugation were found to contain r.b.c.-modifying substances and these could be concentrated and preserved as acetone-dried powders. The powders were quite active; a saline extract of 0.5 mg sensitized 1 ml of a 10% r.b.c. suspension. This activity appeared unaltered after storage for periods of at least 1 year.

The preparations above contained a wide variety of cellular components. Neter's review (11) indicated that the sensitization of washed r.b.c. at room temperature was the result of the adsorption of carbohydrate-containing substances. Attempts to obtain such materials by extraction with cold trichloroacetic acid (0.5 *N* final concentration at 4° C) produced solutions which, after the removal of acid by dialysis, gave strong ring tests but were variable in their ability to modify r.b.c.

Cold trichloroacetic acid extraction applied to Gram-negative bacteria is believed to remove a carbohydrate containing a complex (Boivin antigen) which constitutes the somatic antigen of these organisms. A similar material may be separated from the cells by phenol treatment (11). Recently Kirby (7) showed that a hot phenol extraction removed a glycoprotein from animal tissue and split this so that the carbohydrate moiety was present in the aqueous phase. To apply this technique three volumes of crude sonic extract were mixed with two volumes of melted phenol and incubated with frequent mixing at 60° C for 1 hour. The mixture was cooled, centrifuged to break the emulsion, and the aqueous layer aspirated off and mixed with two volumes of 95% ethanol. The white precipitate which formed was dissolved in saline and reprecipitated twice with ethanol. Saline solutions of this ethanol

precipitate actively sensitized r.b.c. and gave positive Molisch and ninhydrin reactions. On a weight basis it appeared to be about five times as active as crude acetone-dried powders. Serum absorption experiments indicated that the ethanol precipitate contained all the r.b.c.-sensitizing substances present in the crude acetone-dried powders.

The work of Kirby (7) showed that considerable nucleic acid was present in phenol-treated preparations like those described. Absorption spectra of these extracts showed typical peaks at 250–260 mμ characteristic of the nitrogenous bases of the nucleic acids. An attempt was made to remove the latter and to determine the effect on red cell sensitizing capacity. Two-milliliter samples were treated with (*a*) 1 ml of 0.5 *M* $MnCl_2$ (2), (*b*) 1.0 ml of protamine sulphate (10), and (*c*) ribonuclease (5 mg in 1.0 ml of *M*/40 phosphate buffer, pH 7.0, for 1 hour at 37° C). After these treatments the tubes

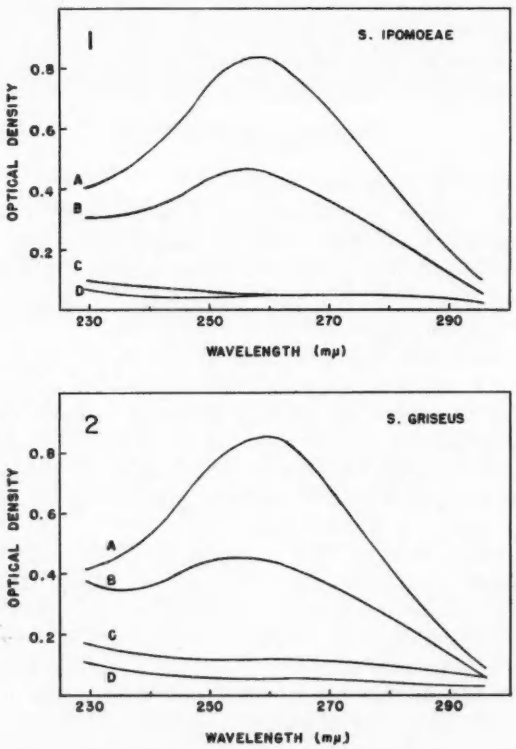

FIG. 1. Absorption spectra obtained from an extract of *S. ipomoeae*. A, original extract; B, ribonuclease-treated; C, $MnCl_2$-treated; D, protamine sulphate treated. Preparations A, B, and C sensitized r.b.c.; preparation D did not.

FIG. 2. Absorption spectra obtained from an extract of *S. griseus*. A, original extract; B, ribonuclease-treated; C, $MnCl_2$-treated; D, protamine sulphate treated. Preparations A, B, and C sensitized r.b.c.; preparation D did not.

were cleared by centrifugation and the r.b.c.-modifying substances reprecipitated with ethanol.

Both $MnCl_2$ and ribonuclease effectively lowered the nuleic acid content of the extracts, as shown in Figs. 1 and 2, but the red cell sensitizing ability appeared unaltered. These data showed no correlation between nucleic acid content and r.b.c.-sensitizing capacity; this was in substantial agreement with the observations of Yokoyama and Hata (17). The results with protamine sulphate (removal of nucleic acid and modifying substances) were anomalous; it is suggested that the very basic protamine salt may have removed polysaccharides by complex formation (borate forms such complexes

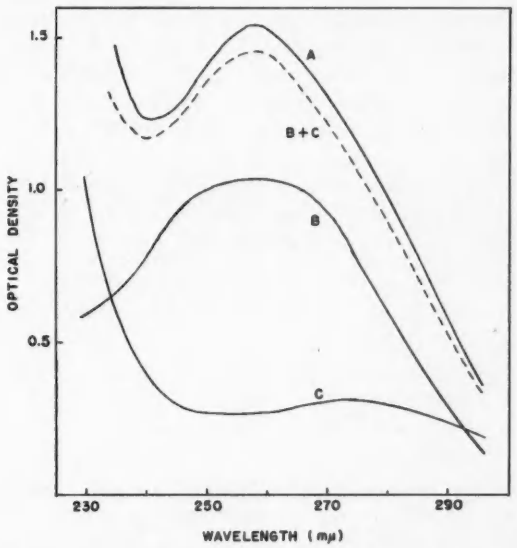

FIG. 3. Absorption spectra of A, supernatant fluid from r.b.c. sensitized with an extract from *S. fradiae*; B, extract of *S. fradiae*; and C, supernatant fluid from unsensitized packed r.b.c. The curve B + C represents the calculated sum of spectra B and C.

under alkaline conditions) along with the nucleic acid precipitate. The absorption spectrum of the supernate from modified red cells was determined and compared to a curve calculated from the sum of the absorption of the original modifying extract plus the absorption of washings from untreated r.b.c. The near coincidence of these curves (Fig. 3) supports the contention that red cell sensitization did not involve the uptake of appreciable amounts of nucleic acid.

Origin of Red Cell Sensitizing Substances

To determine the cellular distribution of the r.b.c.-modifying substances the mycelium was exposed to the action of lysozyme in a buffered saline solution containing sucrose at 0.5 *M* concentration. This procedure resulted in

a fractionation of the mycelium into osmotically sensitive protoplast-like bodies and supernatant (extraprotoplastic) fluid (4). A saline extract of washed packed protoplasts and a portion of the supernatant fluid were compared for their content of red cell modifying substances. The latter were found to be present in the extraprotoplastic fraction as shown in Fig. 4.

The data obtained from this lysozyme fractionation constitute strong evidence that the r.b.c.-sensitizing materials are cell wall constituents. These observations coupled with the work of Romano *et al.* (12, 13) suggest that the substances are mucupolysaccharide in nature. It should be noted that serum absorption experiments (4) suggest the presence of some unextractable red cell sensitizing antigens on the "protoplast" surface.

The red cell modifying antigens present in the acetone-dried powders were not dialyzable nor were they made dialyzable by the action of lysozyme. This result was not totally unexpected since Salton (14) has reported that the products of the action of lysozyme on cell wall substance include components with particle weights of the order of 10,000 to 20,000.

Fluorescein isocyanate tagged antibodies stained mycelium and protoplasts strongly (Figs. 5, 6). Red cells modified with extraprotoplastic substances, in addition to being agglutinated, were also stained by these sera whereas no staining was observed with r.b.c.-treated with protoplast extract.

Cross Reactions and Serum Absorption

In the agglutination tests of Ludwig and Hutchinson (9) cross reactions between species were common (especially with *S. scabies*); attempts to remove these reactions by serum absorptions were unsatisfactory. Similar absorption problems were previously encountered in this laboratory. In the present work it was possible to show the existence of group and specific antigens, as suggested by the above authors, by means of sera absorbed with r.b.c. which had been modified with a heterologous antigen. Table I shows some of the results obtained. These data indicate the existence of antigens of different specificities and also suggest that the "group" antigen(s) may be of quite widespread distribution.

TABLE I

EFFECT OF SERUM ABSORPTION ON SLIDE AGGLUTINATION TESTS

Antigens	Serum			
R.b.c. sensitized with extracts from:	Anti-*ipomoeae*		Anti-*fradiae*	
	Original	Absorbed*	Original	Absorbed*
S. ipomoeae	+	+	−	−
S. fradiae	+	−	+	+
S. albus	−	−	+	−
S. scabies	+	−	+	−
S. griseus	−	−	?	−
S. olivaceous	−	−	+	−

*Absorption with packed r.b.c. sensitized with extract from *S. scabies*.
NOTE: +, agglutination; −, no agglutination.

PLATE I

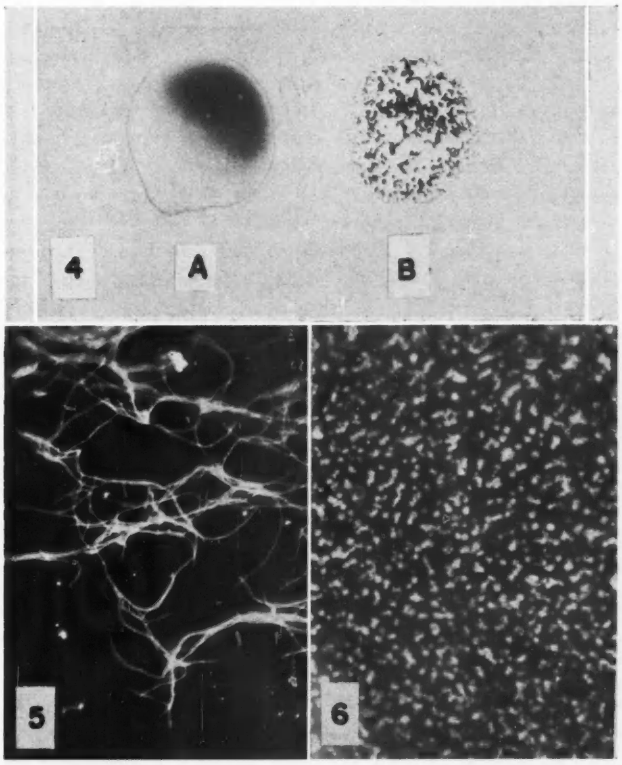

FIG. 4. Mixture of anti-*fradiae* serum with r.b.c. sensitized with "protoplast" extract (A) failed to agglutinate. The antiserum strongly agglutinated r.b.c. sensitized with "extraprotoplastic" substance (B).
FIG. 5. Filaments of *S. fradiae* stained with fluorescein-tagged antibodies.
FIG. 6. "Protoplasts" of *S. fradiae* stained with fluorescein-tagged antibodies.

Discussion

A number of serological techniques of widely varied complexity now exist which are applicable to the streptomycetes. To obtain widespread application a method simpler than complement fixation is definitely required. The technique suggested here is extremely simple and requires only prepared antisera, carrier r.b.c., and an antigen preparation of considerable stability. Reliable results may be obtained by the use of simple slide agglutination tests. Hemagglutination titrations may be performed or the tests converted to hemolytic reactions by the addition of guinea pig complement. The latter procedure renders the test for antibody somewhat more sensitive.

The hypothesis that antigenic components of different specificities are present in the streptomycetes has been confirmed by serum absorption. These observations, however, must be extended to a greater number of species in order properly to assess the distribution of shared antigens. Such a study would do much to indicate the possible value of this serological approach in the classification of actinomycetes.

The potential value of lysozyme in the study of microbial cytology has been emphasized by other workers (14). The use of this enzyme in the present work in conjunction with the description of the properties of the antigen preparations strongly suggest that, for the *Streptomyces*, the passive hemagglutination test is brought about by the reaction of antiserum with antigenic polysaccharides derived from the mycelial cell walls.

Acknowledgments

The authors wish to express their thanks to Dr. C. T. Corke for the photographs and to Dr. D. Waghorne and Mr. J. Berry, Department of Chemistry, O.A.C., for the preparation of the fluorescein amine. They also gratefully acknowledge the valuable technical assistance of Miss Anne Kuhayda.

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AGAR DIFFUSION PRECIPITATION AND COMPLEMENT FIXATION TESTS APPLIED TO THE STUDY OF THE ANTIGENIC RELATIONSHIPS BETWEEN CHROMOGENIC ACID-FAST BACTERIA AND MYCOBACTERIUM TUBERCULOSIS[1]

EDITH MANKIEWICZ

Abstract

The place of two serological techniques, the agar diffusion precipitation reaction and the complement fixation test in the classification of chromogenic acid-fast bacilli, is described. Both techniques reveal evidence of overlapping of antigens as between mycobacteria belonging to pathogenic and to saprophytic strains.

In the past 3 years approximately 4% of the cultures for *Mycobacterium tuberculosis* made in the laboratories of this hospital and found to show evidence of growth of acid-fast microorganisms have contained chromogenic bacteria. Forty-two strains of chromogenic acid-fast bacilli were found among 1060 cultures which were read as positive in the period between February 18, 1955, and December 31, 1956. A study of the antigenic relationships of these strains to each other, to *M. phlei*, and to *M. tuberculosis* will be described in this communication.

The clinical diagnoses of the patients from whose specimens these 42 chromogenic strains of acid-fast bacilli were isolated were as follows: pulmonary tuberculosis, 37; idiopathic pleurisy with effusion, 1; chronic bronchitis and emphysema, 1; pneumoconiosis, 2; and tuberculous bone lesions, 1. The specimens consisted of sputum, fasting gastric contents, bronchial washings, laryngeal swabs, pleural fluid, and pus from bone lesions.

Examination of the records of the 37 patients considered to have pulmonary tuberculosis disclosed (*a*) that in 24 instances typical tubercle bacilli had been isolated from one or several specimens at some time in the past, or were isolated at or about the time the chromogenic strain was found and (*b*) that in 13 instances no acid-fast microorganisms other than chromogenic ones had ever been isolated. In most of these patients chromogenic acid-fast bacilli were isolated from more than one specimen.

The growth characteristics of all strains were studied on a variety of media. Susceptibility to streptomycin, isoniazid, and para-aminosalicylic acid was found to be related to the chemotherapeutic agents given to the patient. In vitro tests performed in order to elucidate the virulence of these bacteria included the following: studies of cord formation in media with and without Tween, cytochemical tests with neutral red and Nile blue, catalase activity, reduction of triphenyltetrazolium, and photochromogenicity. The results of these tests were inconclusive owing to the heterogenicity of the bacterial

[1]Manuscript received May 31, 1958.
Contribution from the Royal Edward Laurentian Hospital, Montreal, Quebec. This project was supported in part by a National Health Grant. Paper read at 25th Annual Meeting of the Canadian Public Health Association, Toronto, on December 10, 1957.

elements which constitute individual colonies. For example, it was found (1) that if cultured in the dark a single chromogenic colony may yield both chromogenic and nonchromogenic colonies, and (2) that after separation solitary photochromogenic colonies may, on subculture, again split into photochromogenic and nonphotochromogenic colonies.

Recourse, in consequence, was made to serological techniques to determine the antigenic nature of chromogenic acid-fast bacteria. Agar diffusion precipitation tests and complement fixation tests were employed.

In 1956, Parlett and Youmans (1) applied the agar diffusion precipitation techniques of Oudin–Ouchterlony to the study of various mycobacteria. They described lines of precipitation which develop in agar gels between wells containing, in alternate order, (*a*) cellular suspensions and (*b*) immune sera produced by rabbits which have been vaccinated with Proskauer culture filtrates of acid-fast microorganisms. The number of lines of precipitation and their distance from the wells were related to the number of specific antigens contained in the cell suspension. Although this procedure yielded significant results in Parlett and Youmans' hands, certain modifications, which it was thought might give additional information, were introduced for the following reasons: (1) nonspecific antibodies, some of them against proteins in Proskauer's medium, develop in animals which have been inoculated with filtrates from cultures grown on this medium; (2) mycobacteria belonging to the saprophytic genus and to the pathogenic genus share some antigens. Group reactions occur which sometimes obscure tuberculospecific reactions. Suspensions of washed, heat-killed bacteria in Freund's adjuvant, instead of culture filtrates, were used for the intravenous injections given to rabbits. After a lapse of 3 weeks and injections of a total of 1.5 mg wet weight of mycobacteria, a certain level of precipitating and complement-fixing antibodies was reached. This level was maintained by subcutaneous injections of 1-mg amounts of mycobacteria at weekly intervals.

The antigens used in both the agar diffusion precipitation tests and the complement fixation tests were of a lipid nature. They were obtained from the bacteria in the following way: The washed cells, along with glass beads, were shaken for 12 hours in a mixture of two parts of 98% ethyl alcohol, one part ether, and 1% concentrated hydrochloric acid. After filtration on fritted glass filters, the extracts were dialyzed against running water for 12 hours. After evaporation an oily substance, which emulsified in saline, was obtained.

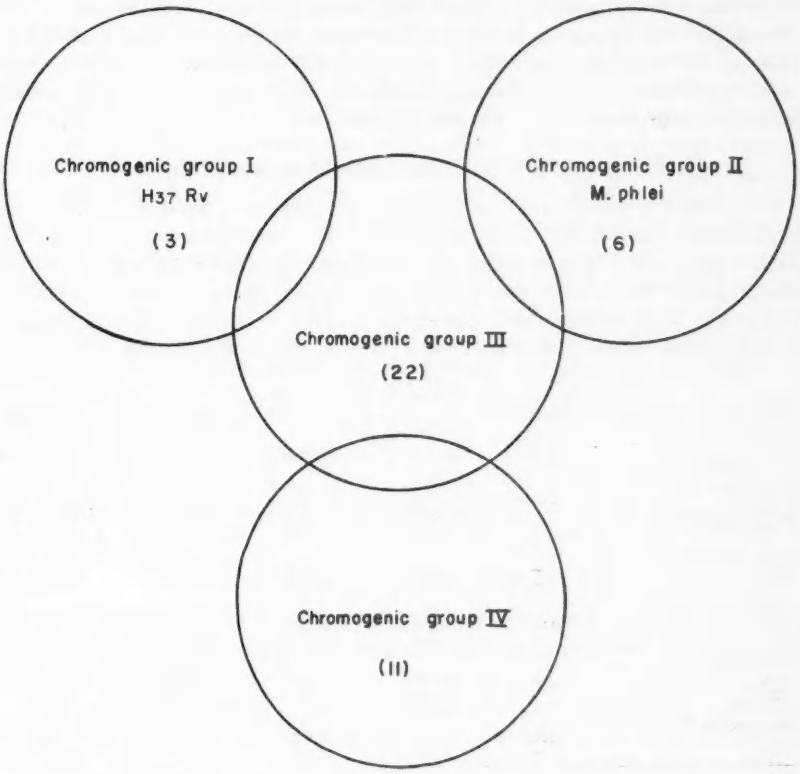
I. Agar Diffusion Precipitation Tests

Lipid antigens from the 42 strains of chromogenic bacteria, from *M. phlei*, BCG, and $H_{37}Rv$, and from three strains of *M. tuberculosis* recently isolated from persons known to have pulmonary tuberculosis were set up against sera obtained from rabbits inoculated with washed cells from one or the other of the following: *M. phlei*, BCG, $H_{37}Rv$, and two strains of chromogenic acid-fast bacteria. These two chromogenic strains were chosen because of

demonstrated differences in pathogenicity. One strain (Z-k), when inoculated in 1-mg wet weight amounts into the subcutaneous tissues of guinea pigs, regularly evoked progressive and fatal disease. The other strain (M-n), when injected into guinea pigs, evoked essentially retrogressive disease.

The technique of double diffusion followed closely the procedure described by Preer (2). An amount of 10 ml of 0.6% agar (containing 0.01% merthiolate, 0.85% saline, 0.2 *M* sodium phosphate adjusted to pH 7.0) was placed in Petri dishes measuring 2 in. in diameter, and was allowed to solidify. Wells were cut into the agar in such a way that a width of 0.6 cm of agar separated one from the other. The position of the bands of precipitation was measured after they became well formed. When strong antigens and antibodies were used, it was possible to take readings after the lapse of 5 hours. As a rule 3 days were required for the formation of all of the lines or bands in one antigen–antibody system. In some instances, lines or bands of precipitation coalesced. When this occurred the antigens were diluted and the tests were repeated. This made it possible, in most instances, to dissociate the bands into lines.

TABLE I



None of the lipid antigens so far employed were absorbed by rabbit sera. Repeated exposure of the antigens to tanned sheep erythrocytes did not reduce their antigenic activity.

It proved to be possible, by counting the lines of precipitation, by measuring their distance from the wells, and by noting the extent of the zone of migration, roughly to classify the chromogenic bacteria as ones related to known virulent mycobacteria (group I, with $H_{37}Rv$ as a representative) and ones related to known saprophytic mycobacteria (group II, with *M. phlei* as representative). Other chromogenic bacteria (group III) were found to share antigens with groups I and II. Still other chromogenic bacteria (group IV) reacted with other chromogens only and not directly with groups I or II.

The accompanying illustration (Table I) depicts the overlapping of these groups. It also shows the distribution, according to groups, of the 42 strains under study.

Experiments have demonstrated (1) that microorganisms which belong to group I, if injected in amounts of 1 mg into guinea pigs, regularly evoke progressive tuberculous disease and (2) that, by contrast, microorganisms which belong to groups II, III, and IV evoke local lesions which, in most instances, promptly resolve. All of the guinea pigs which were injected with microorganisms belonging to group II became tuberculin reactors after the lapse of three or four months. Most of them subsequently became non-reactors. Some of the guinea pigs which were injected with microorganisms belonging to groups III and IV also became reactors. Others did not.

II. Complement Fixation Tests (Kolmer Technique)

In an effort to obtain more precise results, tests using lipid cell extracts as antigens and sera from rabbits inoculated with washed cells, were given a trial. The lipid antigens which had been dialyzed and suspended in saline were used in dilutions in which they were known to be maximally reactive and outside the zone of anticomplementary activity. Lipid antigens prepared in this manner and stored in the cold remain stable for several weeks.

TABLE II

Lipid antigens	Normal rabbit serum	Anti-*phlei* serum	Anti-$H_{37}Rv$ serum	Anti-Z-k chrom. group I serum	Anti-M-n chrom. group II serum
M. phlei	1:64	1:1024	1:128	1:128	1:256
M. tuberculosis $H_{37}Rv$	–	1:256	1:512	1:512	1:128
M. tuberculosis BCG	–	1:256	1:512	1:256	1:256
Chrom. group I					
Z-k	–	1:128	1:512	1:1024	1:256
R-d	–	1:256	1:256	1:512	1:512
Chrom. group II					
M-n	1:16	1:256	1:128	1:256	1:256
S-t	–	1:512	1:128	1:512	1:512
Chrom. group III					
B-n	1:8	1:256	1:64	1:128	1:128
K-l	–	1:128	1:32	1:256	1:128
Chrom. group IV					
D-n	1:16	1:64	1:128	1:256	1:256
N-n	–	1:64	1:64	1:512	1:256

Normal rabbit serum was found to contain antibodies against antigens from *M. phlei.* It was also found to react, to a lesser degree, with antigens from some chromogenic bacteria belonging to groups II and IV.

The complement fixation titers obtained with two representative strains from each of the four groups, as determined by agar diffusion precipitation tests, are recorded in the accompanying table (Table II).

The results were similar to those obtained in the agar diffusion precipitation tests. Complement fixation tests, like the agar diffusion precipitation tests, demonstrate the overlapping of antigens as between typical and atypical tubercle bacilli on the one hand, and saprophytic mycobacteria on the other hand. These tests, however, permit assessment of differences in the proportions of antigens in individual strains.

Discussion

In 1901 Widal and Lesourd (3) and Camus and Pagniez (4) applied Bordet and Gengou's complement fixation reaction to the diagnosis of tuberculosis (5). Since this date many investigators have tried to find a serological technique that might permit of a diagnosis of tuberculosis or, failing this, of assessment of the status of lesions known to be of tuberculous etiology. The following antigens have been recommended at various times: whole desiccated tubercle bacilli, fractions (proteins, polysaccharides, and lipids) extracted from these microorganisms, tuberculin, and tuberculous tissues obtained from man and animals. In recent years erythrocyte-adsorbed antigens have been favored.

Calmette (6) and Nègre and Boquet (7), who tried to diagnose tuberculosis by means of a complement fixation technique, found that the antigen which they employed (an acetone – methyl alcohol extract of tubercle bacilli) did not show absolute specificity in its reaction to humoral antibodies in patients known to have tuberculosis. They noted that acetone – methyl alcohol extracts of tubercle bacilli and of paratubercle bacilli share antigens. They also found that sera of some patients and of some hyperimmunized animals contain inhibitory substances. These observations, along with the fact that as yet no one has determined the role of antibodies in resistance to tuberculous infection, suggest that serological techniques have little to offer in so far as diagnosis and prognosis are concerned.

Serological techniques, however, may offer a means of relating chromogenic acid-fast bacilli either to pathogenic mycobacteria or to saprophytic mycobacteria.

Summary and Conclusions

The results of efforts to assess the antigenic relationships of 42 strains of chromogenic acid-fast bacilli isolated from specimens obtained from patients have been described.

Agar diffusion precipitation tests indicate that chromogenic acid-fast bacilli obtained from human sources can be classified as ones related to virulent mycobacteria (group I, with $H_{37}Rv$ as a representative), ones related

to saprophytic mycobacteria (group II, with *M. phlei* as a representative), and ones that appear to be related, in some respects, to the former and, in other respects, to the latter (group III), or to other chromogenic bacteria only (group IV).

Complement fixation tests carried out with *M. phlei*, BCG, $H_{37}Rv$, and selected strains of chromogenic acid-fast bacilli appear to support the view that the latter can be classified as ones related either to virulent mycobacteria or to saprophytic mycobacteria. They clearly show that the antigens of typical and atypical tubercle bacilli, on the one hand, and of saprophytic mycobacteria, on the other hand, overlap.

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HETEROCARYOSIS AND GENETIC RECOMBINATION IN STREPTOMYCES GRISEOFLAVUS[1]

HIUGA SAITO

Abstract

Upon prolonged incubation of two auxotrophic mutants from *Streptomyces griseoflavus* 142 plated together on minimal medium, heterocaryotic colonies producing only parental type conidia were formed. In addition, from the mixed culture of two double auxotrophs on a medium supplemented with limited amounts of nutrients, stable recombinant-type conidia were recovered at relatively high frequencies. According to these observations, it is suggested that genetic recombination occurs in heterocaryotic mycelial growth antecedent to conidiation. However, complete synapsis appears to be doubtful because of the unbalanced patterns of recombinant recovery.

Introduction

The formation of heterocaryons in hyphae has been demonstrated recently in several species of *Streptomyces* (2, 13). The ability to heterocaryose resembles, at least superficially, that of fungi, although the streptomycetes have many biological characteristics which tend to relate them more closely to bacteria. Among those streptomycetes exhibiting heterocaryosis, only auxotrophic mutants of two species, *Streptomyces coelicolor* (12) and *Streptomyces fradiae* (3, 4), have been found to undergo recombination. However, the information so far reported in the literature is still insufficient to elucidate the mechanism. *Streptomyces griseoflavus* is, as has been reported previously (8), another species in which genetic recombination is observed. The data compiled from intercrosses among several mutant strains together with the finding of heterocaryosis of *S. griseoflavus* will be described in this present communication.

Materials and Methods

Microorganisms

Auxotrophic mutants induced by ultraviolet irradiation in a laboratory strain of *S. griseoflavus* 142 were used throughout the study. Figure 1 is the cell lineage of mutant strains. The details of the mutant induction have already been given elsewhere (9).

Two-letter abbreviations indicate the mutant alleles determining nutritional requirements: *gm* for glutamic acid, *gc* for glycine or serine, *hi* for histidine, *me* for methionine, *tr* for tryptophan, *ad* for adenine, xanthine, hypoxanthine, or uric acid, *ni* for nicotinic acid. Different isolations of mutant characters requiring the same nutrients are distinguished by subscript numbers; possible identities of these markers were not taken into consideration. Capital letters are used to indicate the corresponding wild type alleles.

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Contribution from the Institute of Applied Microbiology, University of Tokyo, Tokyo, Japan.

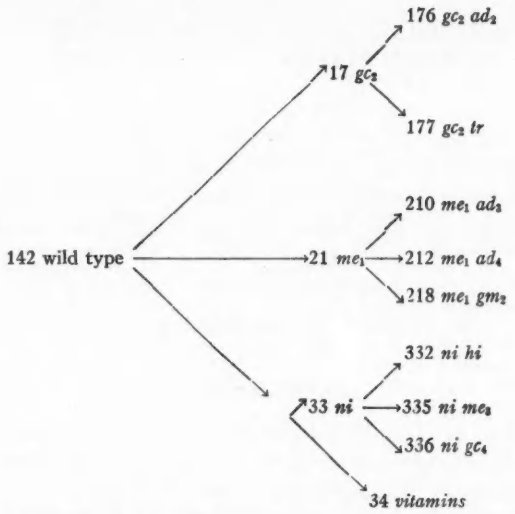

FIG. 1. Lineage of mutant strains. Abbreviations mean nutritional requirements: *gm* for glutamic acid, *gc* for glycine or serine, *hi* for histidine, *me* for methionine, *tr* for tryptophan, *ad* for adenine, etc., *ni* for nicotinic acid. Different origins of the similar mutant characters are distinguished by subscript numbers.

Media

Krainsky's medium with the addition of 0.25% yeast extract and 1% peptone was used for sporulation experiments. Czapek's medium with and without growth-factor supplements was employed as supplemented and minimal media respectively. The latter medium with addition of 0.03% yeast extract and 0.1% peptone served as a limiting medium for crossing experiments. Complete medium consisted of nutrient broth containing glucose. The details of medium preparation have been described previously (9).

Formation of Heterocaryons

Two auxotrophic mutants were cultivated separately on slants of sporulation media for 5 to 7 days at 30° C, at the end of which time the conidia were suspended in 10 ml of sterile saline. After vigorous mechanical vibration for 10 minutes, the suspension was filtered through a Kaburagi's glass filter, G3 No. 2. The suspension was then centrifuged and the sedimented conidia were resuspended in saline. This procedure was repeated twice in order to wash off nutritive contaminants. Finally, the conidial suspension was again filtered through a glass filter, G3 No. 3. With this treatment aggregates of conidia and minute hyphal fragments in the suspension were removed. The filtrate containing individual conidia was diluted so that suspensions were prepared containing 10^5 to 10^8 conidia per ml. The number of conidia in the original suspension were assayed microscopically. From these suspensions 0.1-ml aliquots were removed by pipette and spread on minimal agar plates by means of a glass rod. Aliquots of suspensions from two mutants were spread

simultaneously on the same plate with accompanying control plates. These minimal plates had been dried overnight at 40° to 50° C before use. When the plates were incubated at 30° C for 4 to 6 days, apparent "prototrophic" growth was observed only on the plates upon which two mutants had been inoculated, whereas the control plates, which had been inoculated only with one of either of the mutants, exhibited no growth. The development of "prototrophic" mycelia was somewhat slower on plates with small inocula than on the plates with heavier inocula. About one hundred "prototrophic" colonies developed from 10^6 to 10^7 viable conidia per plate. As will be shown, these colonies were found to be heterocaryons.

Occurrence of Genetic Recombination

Experiments for the genetic recombination in *S. griseoflavus* were carried out in a system similar to that of Sermonti and Spada-Sermonti (12). Two auxotrophic mutants were inoculated on the same agar slant of limiting medium. Possible heterocaryotic growth was observed after 2 to 4 days' incubation at 30° C only in the area where the two mutants mixed (Fig. 2). The conidia produced in this region were harvested after 8 to 12 days' total incubation and a conidial suspension was prepared as previously described. In the present instance, however, the suspension was carefully prepared so that more than 80% of the conidia were single. If many aggregated conidia or mycelial fragments remained in the suspension, they were removed by filtration.

Conidial suspensions containing 10^5 to 10^6 conidia were prepared and plated on minimal and supplemented media. The supplements were added which did not permit the growth of either parental strains, but would allow the growth of recombinants. For example, when 177 gc_2 *tr* and 210 me_1 ad_3 had been crossed, the diluted conidial suspension was spread on five types of media, namely the minimal medium and the medium supplemented with any of the following combinations: glycine and methionine, glycine and adenine, tryptophan and methionine, or tryptophan and adenine. The platings were made in triplicate for each dilution. In parallel, small portions of the suspensions served for the determination of viable counts.

Occasionally, heterocaryotic growth, which might have been evaluated as recombinant colonies, arose after prolonged incubation. Therefore, the recombinant colonies were recorded by marking the reverse side of the glass plate after 72 hours' incubation at 30° C. The plates were incubated for several more days to await conidium maturation, then the nutritional requirements of colonies were determined by the velveteen-replica plating technique. The determination was made only for plates having fewer than 200 colonies. For example, in the above-mentioned cross, the colonies on glycine and methionine supplemented medium were tested for their growth responses on the minimal medium, and on the media supplemented with both, or either of, glycine and/or methionine. After 2 days' incubation, by inspecting the growth responses on these plates, the nutritional requirements of the colonies were determined.

Results

Evidence for Heterocaryosis in Hypha

The "prototrophic" colonies arising from a mixed inoculation of two doubly auxotrophic mutants on the minimal plate generally developed slowly as compared with the wild type or recombinant type colonies. "Prototrophic" colonies, 4–5 days old, consisted largely of mycelial growth which diffused in radial forms over the surface of the medium. These conidiated poorly. After a total incubation of 7 to 10 days, the nuclei in mycelia and conidia of these colonies were harvested in the following manner. A colony was suspended in saline and homogenized in a Waring blender. Conidia and mycelial fragments which resulted were plated on media to establish whether they were "prototrophic" in nature or whether they were auxotrophic. Ten to fifty among 10^3 retained the prototrophic character while the rest of these fragments were auxotrophic. When conidia were harvested from a "prototrophic" colony and plated at the concentration of 10^3 conidia per plate, no prototrophic growth appeared on minimal medium. In this procedure four to seven "prototrophic" colonies were homogenized and every homogenate was plated in duplicate. Where conidia were assayed, aerial growth was removed with an inoculating needle to prepare a suitable suspension. The number of colonies appearing after incubation for 4 days at 30° C was averaged. The results are shown in Table I. This type of experiment was repeated for both the mixed cultures prepared from 210 *me*$_1$ *ad*$_3$ and 336 *ni gc*$_4$ and also 218 *me*$_1$ *gm*$_2$ and 332 *ni hi*. Qualitatively the results obtained were similar and are listed as two separate experiments in the table.

TABLE I

"PROTOTROPHIC"* BEHAVIOR EXHIBITED IN MYCELIAL FRAGMENTS AND IN CONIDIA

"Prototrophic"* colony	Expt. No.	Number of viable cells per 10^3 plating units (on minimal medium)†	
		Homogenized whole colony	Conidia
210 *me*$_1$ *ad*$_3$ +	I	11	0
336 *ni gc*$_4$	II	32	0
218 *me*$_1$ *gm*$_2$ +	I	10	0
332 *ni hi*	II	53	0

*Definition is explained in the text.
†Averaged numbers of four to seven 'prototrophic" colonies.

The conidia which failed to grow on minimal medium were classified as parental types and developed into colonies only on the medium supplemented with the appropriate nutrients. Therefore, it is probable that the "prototrophic" colonies appearing in mixed culture may be ascribed to the formation of heterocaryons which segregate upon conidiation. In rare instances recombinant type growth occurred which appeared to overgrow the heterocaryotic

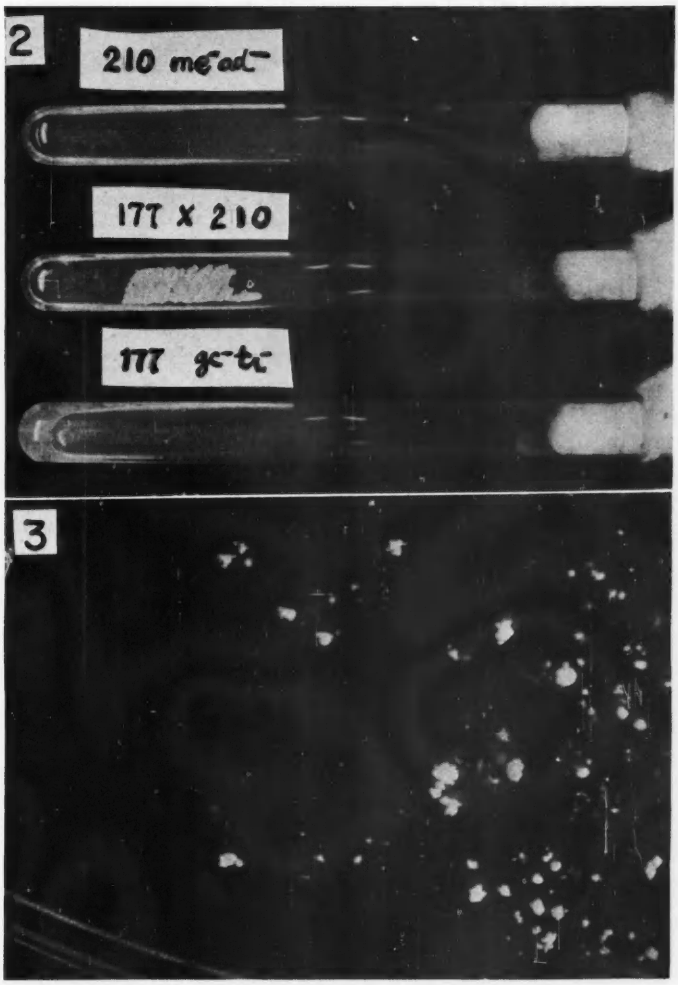

FIG. 2. Mixed culture of 177 gc_2 *tr* and 210 me_1 ad_3 on limiting medium for 12 days at 30° (middle tube). Negative growth of either mutant is shown in top or bottom tube.
FIG. 3. Heterocaryotic growth of 218 me_1 gm_2 and 335 *ni* me_3 on minimal medium for 10 days at 30°. Dense patches indicated by arrow consist of recombinant conidia.

mycelia after incubation for more than 10 days. This event is illustrated as dense colonial growth in Fig. 3 as opposed to the sparse radial forms encountered with "prototrophic" heterocaryons.

In the suspension containing both conidia and mycelial fragments of a "prototrophic" colony between 212 *me*$_1$ *ad*$_4$ and 34 *vitamins* (responds to vitamin mixture), some elements were isolated with a micromanipulator. Among 20 mycelial fragments of 3 to 10 μ in length, 16 failed to grow even in complete medium, and 4 were homocaryotic producing only one type of conidia. Four fragments, which were about 20 μ in length, showed possible branching and segregated into the two parental types. These results might indicate heterocaryosis. However, the possibility that such large fragments might have been contaminated with conidia or small mycelial fragments of another type could not be excluded. The failure of short fragments to grow might be due to their enviable nature after long incubation.

Characterization of Genetic Recombination

Genetic recombination described in Materials and Methods occurred between two doubly auxotrophic mutants inoculated together on limiting medium. Among the resulting recombinant conidia those carrying at least two wild type alleles were selected. Segregation patterns of unselected markers of the cross between 177 *gc*$_2$ *tr* and 210 *me*$_1$ *ad*$_3$ are shown in Table II. Such crosses were carried out among six combinations of auxotrophic mutants. The data for each cross are shown in Table III. The kinds of recombinants recovered were similar for separate experiments for a cross but the frequencies were somewhat variable. Recombinants involving two marker characters were isolated and verified in subculture.

TABLE II

SEGREGATION PATTERNS OF UNSELECTED MARKERS IN SELECTED RECOMBINANTS

Cross (parents)	Selected recombinant	Segregation at other loci			
		Parental type		Recombinant type	
	ME$_1$ *GC*$_2$	*tr* *AD*$_3$ 40	*TR* *ad*$_3$ 0	*tr* *ad*$_3$ 0	*TR* *AD*$_3$ 28
177 *gc*$_2$ *tr* *ME*$_1$ *AD*$_3$ ×	*ME*$_1$ *TR*	*gc*$_2$ *AD*$_3$ 0	*GC*$_2$ *ad*$_3$ 0	*gc*$_2$ *ad*$_3$ 0	*GC*$_2$ *AD*$_3$ 114
210 *GC*$_2$ *TR* *me*$_1$ *ad*$_3$	*AD*$_3$ *GC*$_2$	*tr* *ME*$_1$ 22	*TR* *me*$_1$ 4	*tr* *me*$_1$ 0	*TR* *ME*$_1$ 11
	AD$_3$ *TR*	*gc*$_2$ *ME*$_1$ 0	*GC*$_2$ *me*$_1$ 46	*gc*$_2$ *me*$_1$ 0	*GC*$_2$ *ME*$_1$ 95

The homogeneity of conidia in a recombinant colony was examined as follows. A large number of conidia were removed from a "recombinant" colony with the aid of a platinum inoculating needle, suspended in saline, and inoculated onto complete agar. The nutritional requirements of these "secondary"

colonies were evaluated by the replica plating technique employing minimal medium and five types of supplemented media which would detect each type of recombinant from the cross. For example, in the case of 176 *gc*$_2$ *ad*$_2$ × 335 *ni me*$_3$, 14 types of recombinants are possible which can be determined on five media supplemented with GAN, GAM, GNA, ANM, and GANM (G: glycine, A: adenine, N: nicotinic acid, M: methionine). Growth was observed after incubation at 30° C for 2 days.

TABLE III

FREQUENCIES OF SELECTED RECOMBINANTS AMONG AUXOTROPHS

Cross (parents)	Expt. No.	Number of conidia: Total tested	Number of conidia: Recombinant*		Cross (parents)	Expt. No.	Number of conidia: Total tested	Number of conidia: Recombinant*	
177 *gc*$_2$ *tr*	I	1×10^5	+	120	176 *gc*$_2$ *ad*$_2$	I	1×10^5	+	4
			tr	210				*ni*	146
×			*me*	40	×			*ad*	184
								me	22
210 *me*$_1$ *ad*$_2$	II	1×10^8	+	4	335 *ni me*$_3$			*ad ni*	4
			me	38				*ad me*	1
			tr	1					
177 *gc*$_2$ *tr*	I	1×10^6	+	0	177 *gc*$_2$ *tr*	I	1×10^6	+	330
			hi	298				*ni*	135
×			*tr*	34	×			*me*	28
								tr	2
332 *ni hi*	II	5×10^6	+	0	335 *ni me*$_3$			*tr me*	1
			hi	823		II	7×10^7	+	430
			tr	17				*ni*	177
								me	56
								tr	23
210 *me*$_1$ *ad*$_2$	I	3×10^7	+	0	210 *me*$_1$ *ad*$_3$	I	1×10^8		0
			me	66					
×			*hi*	15	×	II	1×10^8		0
			me ni	3					
			ad hi	1		III	1×10^6	+	0
332 *ni hi*			*me hi*	1	336 *ni gc*$_4$			*me*	3

*The symbol "+" designates a prototrophic type.

As shown in Table IV, 51 "recombinant" colonies studied were found to show no segregation on conidiation and differed from the "prototrophic" colonies ascribed to the formation of heterocaryons. On the other hand, among "recombinant" colonies selected on supplemented media 22 out of 53 were heterogeneous, producing conidia of one recombinant type together with either parental type. It is probable, however, that the occurrence of these heterogeneous colonies was due to the formation of a heterocaryon between the recombinant and parental mycelial growth. Two colonies indicated by asterisks in Table IV segregated two recombinant types and a parental type. The heterogeneity of these colonies might be due to the development of a heterocaryon by two recombinant conidia which were plated by chance in juxtaposition. A more probable explanation is the presence of heterozygous and/or heterocaryotic conidia.

TABLE IV

SEGREGATION IN RECOMBINANT COLONIES WITH CONIDIATION

Cross	Recombinant colony tested: Medium (supplement)	Type†	Number	Type† (number) of conidia found on the colony
177 *gc*$_2$ *tr* × 210 *me*$_1$ *ad*$_3$	Minimal	+	11	+ (all)
176 *gc*$_2$ *ad*$_2$ × 335 *ni me*$_3$	Minimal	+	9	+ (all)
	Aden. niac	*ad*	4	*ad* (all)
	Aden. niac	*ni*	4	*ni* (all)
	Aden. meth	*ad*	3	*ad* (all)
	Aden. meth	*ad*	1	*ad* (7), *ni me* (6)
	Aden. meth	*ad*	1*	*ad* (20), *ni* (8), *ni me* (10)
	Aden. meth	*me*	1	*me* (all)
	Aden. meth	*me*	1	*me* (8), *gc ad* (7)
	Aden. meth	*me*	1*	*me* (10), *ni* (2), *gc ad* (10)
177 *gc*$_2$ *tr* × 335 *ni me*$_3$	Tryp. meth	+	1	+ (all)
	Tryp. meth	+	3	+ (83), *ni me* (22)
	Tryp. meth	*tr*	1	*tr* (50), *ni me* (22)
	Tryp. meth	*me*	1	*me* (all)
	Tryp. meth	*me*	4	*me* (96), *ni me* (75)
	Tryp. meth	*me*	2	*me* (57), *ni me* (21), *gc tr* (36)
210 *me*$_1$ *ad*$_3$ × 332 *ni hi*	Meth. hist	*hi*	6	*hi* (all)
	Meth. hist	*me*	1	*me* (all)
	Meth. hist	*me*	2	*me* (49), *ni hi* (25)
	Meth. hist	*me*	1	*me* (120), *me ad* (45), *ni hi* (35)
	Meth. niac	*me*	1	*me* (all)
	Meth. niac	*me*	1	*me* (21), *ni hi* (15)
	Meth. niac	*me ni*	3	*me ni* (all)
177 *gc*$_2$ *tr* × 332 *ni hi*	Glyc. hist	*hi*	4	*hi* (all)
	Glyc. hist	*hi*	1	*hi* (182), *ni hi* (28)
	Tryp. hist	*hi*	2	*hi* (all)
	Tryp. niac	*tr*	3	*tr* (77), *ni hi* (65)

*These colonies segregated two recombinant types and a parental type.
†The symbol "+" designates a prototrophic type.

Another possibility to account for observed recombinants is the reversion of auxotrophic markers. Attempts were made to exclude this interpretation. Analyzing conidia harvested from cultures on media supplemented with various concentrations of nutrients, I found the reversion frequencies of *me*$_1$ and *hi* to be 1×10^7 or less per conidium, and detected no spontaneous reversions with other markers. If these reversions occurred during the course of the experiment, the recovery of *ad*$_3$-type conidia from 210 *me*$_1$ *ad*$_3$ and of *ni*-type conidia from 332 *ni hi* might be expected. However, the data of Table III indicate that no reversion of the *me*$_1$ locus or the *hi* locus was observed under the experimental conditions. Moreover, the occurrences of prototrophic type conidia from crosses such as 177 *gc*$_2$ *tr* × 210 *me*$_1$ *ad*$_3$ were much too frequent to be dependent upon double reversions at two loci. The recovery of double auxotrophs such as with 210 *me*$_1$ *ad*$_3$ × 332 *ni hi* showing non-parental combination of nutritional requirements indicates genetic recombination obviously.

Discussion

The formation of a heterocaryotic state in mycelia developed from two auxotrophic mutants was reported in *S. griseus* and *S. cyaneus* by Bradley and Lederberg (2); in *S. griseus*, *S. albus*, and S. *venezuelae* by Szybalski and Braendle (13); tentatively in *S. coelicolor* by Sermonti and Spada-Sermonti (12). Bradley (1) has reported heterocaryotic or heterozygotic conidia in a strain of *S. coelicolor*.

The experimental results presented in this paper indicated heterocaryon formation in the mycelial growth of *S. griseoflavus*. This conclusion was based on the following: (1) "Prototrophic" colonies were formed in a mixed culture of two auxotrophic mutants on minimal medium; (2) "prototrophic" colonies, which at 4 to 5 days consisted of mycelial growth, produced two parental type conidia upon continued incubation; (3) the "prototrophic" behavior of the colonies appeared to be inherited only by mycelial transfer; (4) during the course of experiments designed to detect genetic recombination, mosaic type colonies which segregated upon conidiation into parental and recombinant types were encountered; (5) "prototrophic" mycelia removed by means of a micromanipulator appeared to be heterocaryotic fragments.

Although genetic recombination was demonstrated, no conclusive evidence is presented for the mechanism. However, several possibilities may be expressed and will be discussed below.

Hopwood (5) found that all possible recombinants could be recovered in a cross of two doubly auxotrophic mutants of *S. coelicolor* (this strain was different from that used by Sermonti and Spada-Sermonti (12) and by Bradley (1)). He speculated that the mechanism would be occasional nuclear fusion followed by reduction division. In the recombination studies with *S. fradiae* by Braendle and Szybalski (3) it was proposed that unstable diploid fusion nuclei segregate haploid recombinants. Their hypothesis was based on observations of "prototrophic" colonies which yielded parental as well as recombinant conidia.

With *S. griseoflavus* the conidium consists of a diploid or bipartite nucleus as reported previously (10, 11). However, the nuclei were homozygous regardless of the mixture of nuclei in the mycelial phase because duplication in the conidium was shown to result from self-replication of a single nucleus. Under these conditions it seems improbable that genetic recombination occurs within the conidia.

As indicated previously, the detection of "recombinant" colonies segregating two recombinant type conidia along with the parental types may suggest that unstable heterozygotes are formed by the fusion of dissimilar nuclei in the heterocaryon. These heterozygous nuclei would undergo segregation before conidiation so that homozygous conidia producing homogeneous colonies result. The possibility exists that conidiation may precede segregation resulting in an occasional heterozygous conidium. However, the segregation patterns of the heterogeneous "recombinant" colonies had a

ratio different from that expected by reciprocal recombination in a heterozygous diploid nucleus. A fractional heterozygote as described by Lederberg (6) might be postulated to explain the observations.

It is probable that genetic recombination occurs in the heterocaryotic hyphae. The dense growth attributed to recombination appeared on areas of the radial sparse growth of heterocaryotic mycelia (Fig. 3). According to the classical conception of genetic recombination, reciprocation should result following meiosis. If this were the case, it might be expected that, in a cross of two double auxotrophs contributing four heterozygous loci, where two markers were used for selection, the other two characters should segregate independently. Such an analysis is discussed for random samples of ascospores in *Aspergillus nidulans* (7). The observed ratios shown in Table II do not agree with theoretical values and furthermore the imbalance was too unpredictable to elucidate the linkage relationships of the markers employed.

Recombinant nuclei once formed may be selected during the growth and division which precede conidiation. The result determined by the environmental conditions imposed by the minimal nutrition would be an excess of prototrophs and a diminution of double auxotrophs. Furthermore it is surprising that some types of conidia which had single requirements were never detected among the recombinants. Such a loss in the segregation pattern is not without precedent in *Streptomyces* having been observed in *S. coelicolor* (12) and *S. fradiae* (4). Four possible explanations of this loss may be given: (1) reversion of the partner marker, (2) selection during nuclear division, (3) differential viability, (4) and/or actual loss at segregation, being due to an extraordinary mechanism. Among these four possibilities the fourth is the most compatible with the experimental results. To understand the mechanism of genetic recombination in this genus, the absence or presence of single auxotrophs may be more important than recombinant frequencies.

The reciprocal cross-over mechanism being adopted, if the three loci *hi/HI tr/TR*, and me_1/ME_1 are closely linked, the recombination between 177 gc_2 *tr*, 210 me_1 ad_3, and/or 332 *ni hi* may be expected to result predominantly in *tr*-, *me*-, and *hi*-single recombinants, according to the selection procedure employed. The experimental results shown in Table III are in good agreement with this expectation. However, the cross between 335 *ni* me_3 and 177 gc_2 *tr* or 176 gc_2 ad_2 cannot be interpreted by reciprocal recombination. The four markers in either cross should segregate independently but only three types of single auxotrophs were detected. The *gc*-type was never encountered in single or double auxotrophic recombinants. The formation of a fractional heterozygote would explain all the results and this is tentatively proposed.

In the cross between 210 me_1 ad_3 and 336 *ni* gc_4 no recombinants were recovered. This may be ascribed to lack of compatibility between two types of nuclei or to the suppression of recombination, since it had been shown that these mutants could easily form balanced heterocaryons. Such behavior might explain the failure to demonstrate genetic recombination with other

species of *Streptomyces* (2). In addition, it should be emphasized that the absence of prototrophic recombinants in some crosses does not necessarily reflect the inability of undergoing recombination. This was observed in the crosses 210 *me*$_1$ *ad*$_3$ × 336 *ni gc*$_4$, 177 *gc*$_2$ *tr* × 332 *ni hi*, and 210 *me*$_1$ *ad*$_3$ × 332 *ni hi*.

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PRODUCTION OF OB DIAGNOSTIC ANTISERA FROM MOTILE ESCHERICHIA COLI STRAINS[1]

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Abstract

Experiments have been carried out on the reduction of H antigenicity of motile *Escherichia coli* strains for use in the production of OB diagnostic antisera when naturally occurring non-motile variants are unavailable.

Using homologous H agglutinins and/or phenol, H-inagglutinable "variants" were produced, from which OB suspensions were prepared. Rabbits immunized with these suspensions yielded antisera which met the requirements of *E. coli* OB diagnostic antisera.

The characteristics of the experimental antisera prepared are discussed briefly, and some advantages of the methods presented over others commonly used in the laboratory practice are indicated.

Introduction

The serological identification of *Escherichia coli* (hereafter referred to as *E. coli*) strains requires the provision of an extensive series of *E. coli* diagnostic antisera (5). Among these, OB sera are of special importance, since many of the pathogenic *E. coli* strains, especially those associated with infantile diarrhea, possess a B type of surface (K) antigen (3, 7, 11).

The basic method of preparing *E. coli* OK (OB) antisera consists of immunizing rabbits with well-developed K (B) forms of preferably non-motile (aflagellar) strains possessing the required O and K (B) antigens (2, 4, 8). Since non-motile strains of a given serologic type may be unavailable, however, and since there are *E. coli* strains for which non-motile variants have not yet been reported, we are sometimes forced to use flagellated strains for the preparation of OK (OB) antisera.

In such cases several methods have been recommended. Kauffmann (9) has suggested the use of living cultures (suspensions in salt solution) from 20-hour agar plates as immunizing antigen. Although, of course, the antisera prepared in this way do contain H agglutinins, Kauffmann states that "no H agglutination appears in agglutination tests with 20-hour agar cultures". In a critical investigation of this statement, Stuart and Carpenter (13) examined the H antigen production of 62 *E. coli* strains and found that 16 antisera prepared from motile strains by Kauffmann's method yielded 53 positive agglutination reactions of a pure H type when tested with living 20-hour agar cultures. The experiences of Wright and Villanueva (14) also differed from that of Kauffmann. In a study on the motility of 100 *E. coli* strains, they checked 43 strains for H agglutinability in K suspensions and 2 of them for H antigenicity in preparing K antisera by the method of Kauffmann. Motility was observed in about two thirds of the 20-hour agar cultures and H agglutinability in about one half of the K suspensions. All three K antisera

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prepared with one of the motile strains showed relatively high H titers varying from 1:6400 to 1:51,200. They recommend checking the immunizing K suspension for the desired absence of H antigen, since, if this is well developed in the K suspension, the H reaction of the serum may override and mask the K reaction. Edwards and Ewing (2) offered three different methods for preparing *E. coli* OB (OK) antisera using non-motile strains, whenever possible, or freshly isolated strains that have not been passaged in semisolid medium. One of these methods requires the use of 4- to 5-hour-old broth cultures as immunizing antigen. In the second one, bacterial suspensions prepared from 12- to 14-hour-old growth on agar slants are to be employed. The third one is a modification of Roschka's method (drying the bacterial suspension in acetone after treatment with alcohol). When any of these methods are employed using motile strains, however, the resulting OK antisera may contain H agglutinins in considerable amounts. These may cause confusion in reading the results of the agglutination tests, unless they have been eliminated by the procedure of serum absorption.

In an attempt to find a method of producing from flagellated strains OB antisera with negligible amounts of H agglutinins, experiments have been carried out on the reduction of the H antigenicity of originally highly motile *E. coli* strains. The principles underlying this experimental work are supported by data in the literature dealing with the effect of the homologous H antibodies and that of the phenol on the motility and the flagellar morphology of some Gram-negative bacteria.

Gard (6) demonstrated the immobilizing effect of homologous H agglutinins on a motile coliform strain possessing a Salmonella H antigen. Menolasino, Goldin, and Hoffman (10) found that Gram-negative bacteria of various types of flagellation, transitorily immobilized when grown in broth containing specific H agglutinins, showed significant shortening and/or loss of curvature of the flagellae. Braun (1) stated that *Proteus* bacteria grown on solid media with 0.1% content of phenol lost their motility. After the bacteria were transferred to ordinary media without phenol content, however, their motility was re-established. In a study of the flagellation and motility of *Aerobacter cloacae* and *E. coli* strains, Smith (12) found that the addition of phenol to the culture medium resulted in loss of motility due to inhibition of the production of the flagellae.

On the basis of these data, attempts were made to produce non-motile *E. coli* cultures of impaired flagellation in the hope of reducing H antigenicity, but, if possible, without causing any damage to the somatic and, particularly, to the surface antigens. This paper presents the results of these experiments applied in the preparation of *E. coli* OB diagnostic antisera.

Materials and Methods

Bacterial Strains

For experimental purposes, two actively motile *E. coli* strains, for which non-motile variants were unavailable, were employed: the stock strain C 130, i.e. the Canioni strain with the antigenic structure O125:K70(B15):H19, and

the stock strain C 131, originally named E 611 with the antigenic formula O126:K71(B16):H2. Previously, the strains had been thoroughly checked for purity, for motility, and for cultural, biochemical, and serological characteristics. The findings were in accordance with the known properties of the given *E. coli* types. It should be emphasized that non-motile variants could not be detected in the population of the original stock strains.

For control purposes, highly motile *E. coli* stock strains possessing the same flagellar antigens but different somatic antigens from those of the experimental strains were used: the stock strain EC 484 with H19 flagellar antigen and unknown somatic antigens, and the stock strain EC 501 with the antigenic formula O55:K59(B5):H2.

Culture Media

The following culture media were employed in the experiments: (1) veal infusion broth and (2) veal infusion agar prepared in the usual way; (3) 0.2% semisolid agar in Craigie tubes, with and without 0.1% phenol content; (4) thiotone agar plates prepared according to the Baltimore Biological Laboratory's formula for blood agar base containing 1% thiotone, with and without 0.1% phenol content; (5) phenolized agar plates prepared from veal infusion agar with the addition of 0.1% phenol (all the plates being used in Petri dishes after being dried for ½ hour at 37° C).

Agglutination Tests

Veal broth cultures cultivated at 37° C for 4 to 6 hours or bacterial suspensions prepared from overnight growth at 37° C on agar plates were used as antigens in live state for B and H agglutinations, and boiled at 100° C for 1 hour for O agglutination. For the demonstration of the presence or absence of *E. coli* O, B, and H antigens in the cultures or suspensions of the experimental and control strains, *E. coli* monovalent diagnostic antisera (factor sera), prepared previously and used for routine diagnostic purposes in our laboratory, were employed.

Tube agglutination tests were performed in small test tubes (3×½ in.) by the use of serial doubling dilutions of the sera in amounts of 0.5 ml in each tube, with the addition of the same amounts of the antigen containing approximately two million organisms per milliliter. The tests were carried out and read according to the appropriate techniques used for the O, B, and H agglutinations. The highest dilution of the sera which agglutinated approximately fifty per cent of the bacteria in a typical manner was considered as the final titer of the sera. Slide agglutination tests for B agglutination were performed on precleaned microscope slides with low dilutions of the sera (1:2–1:25), according to the usual technique. Only typical reactions occurring immediately, i.e. within a few seconds, were considered as positive.

Motility Tests

Motility tests were performed in parallel by microscopic examination of hanging drop preparations and by cultivation at 37° C in semisolid agar with and/or without 0.1% phenol content.

Methods for Production of Immunizing Antigens

E. coli H2 and H19 factor sera respectively was added aseptically to veal infusion broth in tubes to make final concentrations of 0.5 to 4%. After having been checked for sterility by overnight incubation at 37° C, the tubes were stored at 5° C until used. Smooth, O-inagglutinable, good B forms were selected from colonies of both experimental stock strains grown on thiotone agar plates, after they had attained high motility and fairly good H agglutinability, following serial passage on semisolid agar. From such colonies of each experimental strain, inoculations were made into veal infusion broth containing homologous H antibodies. After incubation at 37° C for 4 to 6 hours, the broth cultures were refrigerated, and at weekly intervals, inoculations were made from the upper part of the broth cultures onto phenolized agar plates. After incubation at 37° C for 24 to 48 hours, sweeps and single colonies of S form were examined for motility and for O, B, and H agglutinability. When most of the colonies tested proved to be non-motile by microscopic examination and by cultivation in phenolized semisolid agar, O-inagglutinable colonies of good B, but reduced (preferably lost) H agglutinability, were selected and transferred onto fresh phenolized agar plates. This procedure was repeated, if necessary, until the entire growth on phenolized agar plates proved to be H-inagglutinable in the starting dilution (1:20–1:40) of the homologous H factor serum. From this non-H-agglutinable "variant", immunizing antigen was prepared by harvesting the growth of the "variant" on phenolized agar plates either suspending the bacteria in veal infusion broth or using the method of Roschka according to Edwards and Ewing (2).

A simplified method for producing H-inagglutinable antigen from motile *E. coli* cultures consisted of the same procedure except that the preliminary process, involving the use of homologous H antibodies, was omitted. In this case, O-inagglutinable good B forms of the highly motile experimental strains were cultivated directly on phenolized agar plates. Single colonies, which on examination revealed loss of motility and reduced or (preferably) lost H agglutinability, were selected. Immunizing antigen was prepared as described above, when after a few additional passages on phenolized agar plates, the entire growth, when tested by tube agglutination, gave a negative reaction in the starting dilution of the homologous H factor serum.

Preparation of Rabbit Immune Sera

Male rabbits weighing about five pounds were immunized after their sera had been checked for the absence of natural antibodies, which might react with the H antigens of the experimental and control strains. Only rabbits with no demonstrable amounts of such antibodies were employed. The rabbits were injected intravenously, at intervals of 3 to 4 days, with increasing amounts (from 0.25 to 4.0 ml) of the appropriate antigen. A trial bleeding was performed 5 days after the fifth injection, and the B titer of the serum was determined by tube agglutination test. Depending upon the

results, the rabbits were either bled out, or given additional injections, until a satisfactory titer was obtained. For control purposes, *E. coli* OB antisera were prepared with the same experimental strains according to the methods suggested by Edwards and Ewing (2). All the sera were preserved with 0.01% merthiolate and stored at 5° C.

Experimental Results

Production and Evaluation of E. coli O125:K70(B15) Antisera

The stock strain C 130 with the antigenic structure O125:K70(B15):H19 was passaged three times on thiotone agar plates with subsequent selection and transfer of O-inagglutinable, smooth colonies of good B agglutinability, then passed five times through semisolid agar for enhancement of its motility. At the end of this procedure, the strain C 130 was agglutinated in the living state by the B15 (O125:K70) factor serum in a final dilution of 1:400 and by the H19 factor serum in a final dilution of 1:3200. The O125 factor serum did not agglutinate the living bacteria in dilutions higher than 1:20, but showed a titer of 1:6400, when tested with a 6-hour broth culture of the strain heated at 100° C for 1 hour.

In this state, the strain C 130 was inoculated into veal infusion broth containing 1% homologous (H19) factor serum, incubated at 37° C for 6 hours, and then refrigerated at 5° C. For control purposes, veal infusion broth without addition of H antiserum was inoculated, incubated, and stored in the same way. At weekly intervals, inoculations were made from the upper part of the broth cultures onto phenolized agar plates, and after incubation at 37° C for 24 to 48 hours, sweeps and single colonies were examined for motility and also for O, B, and H agglutinability. At the end of the fourth week most of the single colonies examined proved to be non-motile. The H19 factor serum still agglutinated the subculture of the selected non-motile "variant" grown on phenolized thiotone agar plate in a final dilution of 1:200, whereas the control gave a positive reaction in a final dilution of 1:3200. By means of serial transfers on phenolized thiotone agar plates, the H agglutinability of the non-motile "variant" was further reduced, until finally after the fourth passage the "variant" proved to be H-inagglutinable in the starting dilution of the H19 factor serum. A satisfactory B agglutinability with a final titer of 1:640 was maintained, and an insignificant O agglutinability (1:40) was present, when tested in the living state. After incubation at 37° C for 24 hours, the growth of this "variant" on several phenolized thiotone plates was harvested and suspended in 95% alcohol for preparation of dried antigen by the method of Roschka. Two rabbits (Nos. 229 and 358) were immunized with this antigen as outlined above. The antisera were tested for the presence of O, B, and H agglutinins in tube and slide agglutination tests with heated and living cultures, respectively, of the homologous experimental (C 130) and control (EC 484) strains. The antigenic properties of these control strains were determined, at the same time, by the use of the appropriate O, B, and H factor sera (Table I).

TABLE I

TESTING OF *E. coli* O125:K70(B15):H19 ANTISERA

Antisera		Type of agglutination	Final titers: In tube test: With boiled cultures: C 130	With boiled cultures: EC 484	In tube test: With living cultures: C 130	With living cultures: EC 484	In slide test: With living cultures: C 130	With living cultures: EC 484
Factor	O 125	O	6,400	–	20	–	–	–
	B 15	B	.	.	400	–	8	–*
	H 19	H	.	.	3,200	6,400	25	25
Experimental	No. 229	O	5,120	–	20	–	–	–
		B	.	.	100	–	6	–*
		H	.	.	–	–	–*	–*
	No. 358	O	10,240	–	20	–	–	–
		B	.	.	640	–	16	–*
		H	.	.	–	–	–*	–*
Control	No. 307	O	12,800	–	20	–	–	–
		B	.	.	?	–	?	–*
		H	.	.	6,400	3,200	25	25

NOTE: – = negative in a dilution of 1:20.
–* = negative in a dilution of 1:2
. = not tested.
? = titer could not be exactly determined.
C 130 = *E. coli* strain with the antigenic structure O125:K70(B15):H19.
EC 484 = *E. coli* strain with the antigenic structure O?:K?:H19.

Both experimental antisera (Nos. 229 and 358) contain great amounts of O agglutinins, which are revealed, however, only when tested with boiled cultures of the experimental (homologous) strain. Living cultures of the same did not exhibit O agglutination in a dilution higher than 1:20. Experimental serum No. 229 has a rather low B titer, when tested in tube agglutination test with the living culture of the homologous strain (C 130), but it is still usable in slide agglutination tests for the purpose of live "spot" B agglutinations in a final dilution of 1:6. Experimental serum No. 358 possesses a much stronger B titer and, consequently, it permits satisfactory "spot" tests in the considerably higher dilution of 1:16. In order to check the experimental antisera for the possible presence of H19 agglutinins, the control strain EC 484 possessing the same (H19) flagellar antigen, but different somatic antigens from those of C 130, was used. The living culture of the control strain was agglutinated in tube agglutination test by the H19 factor serum in a final dilution of 1:6400, and in slide test it gave a strong reaction of pure H type in a dilution of 1:25, which indicates the high H19 antigenic content of the control strain. In spite of this, both experimental antisera failed to agglutinate the living cultures of the control strain EC 484, when tested in tube agglutination test even in the very low dilution of 1:20 and in slide agglutination test in a dilution of 1:2. This provides clear evidence of the absence of significant amounts of H19 agglutinins in the OB antisera produced.

For purposes of comparison, OB antiserum was prepared with the same experimental strain (C 130), according to one of the three methods suggested by Edwards and Ewing (2). The stock strain C 130 was prepared for the production of the immunizing antigen in the same manner as described above, except that transfers through semisolid agar were not undertaken, and consequently, the strain was in a state of considerably less vigorous motility and lower H agglutinability (1:640). One rabbit (No. 307) was immunized with suspensions made from overnight (approximately 15 hours old) growth on veal infusion agar plates. The antiserum thus obtained (No. 307) was tested in the same way as mentioned before. The results of tube and slide agglutination tests (Table I) indicate that this control serum contains relatively large amounts of H19 agglutinins, and as a consequence, the B titer of the serum cannot be exactly determined for either tube or slide agglutination tests.

Production and Evaluation of E. coli O126:K71(B16) Antisera

After a selective procedure referred to above was performed, the actively motile stock strain C 131 with the antigenic formula O126:K71(B16):H2, when tested with the appropriate factor sera, revealed the following serological characteristics: O agglutinability, as a live antigen—1:40, after heating at 100° C for 1 hour—1:6400; B agglutinability 1:800; H agglutinability 1:8000.

In this state, the strain C 131 was passaged at weekly intervals (after incubation at 37° C for 6 hours and storage at 5° C) in veal infusion broth containing doubling amounts (from 0.5 to 4.0%) of homologous (H2) factor serum. Control passages in absence of specific H antibodies were included. After the fourth passage, a non-motile, H-inagglutinable "variant" was selected and transferred to phenolized agar plates. The growth from these was suspended in veal infusion broth and used as an antigen for the immunization of a rabbit (No. 344).

In a simplified procedure, the same experimental strain (C 131) was cultivated serially on agar plates containing 0.1% phenol, with selection and transfer of single colonies, which demonstrated reduced motility and H agglutinability. After the third passage, one of the H-inagglutinable "variants" thus obtained was used for preparation of antigen as outlined above, for the immunization of another rabbit (No. 345).

In addition, two control antisera were produced with the same experimental strain (C 131), but in a state of slight motility and low H agglutinability (1:800). For the preparation of the antigens, the other two methods recommended for use in laboratory practice (2) were employed. Rabbit No. 343 was immunized with a 6-hour-old broth culture of strain C 131. Rabbit No. 206 was injected with an antigen prepared by the method of Roschka.

All four antisera (Nos. 344, 345, 343, and 206) were tested for O, B, and H agglutinin content with cultures heated at 100° C for 1 hour and living cultures, respectively, of the homologous experimental (C 131) and control

TABLE II

TESTING OF *E. coli* O126:K71(B16):H2 ANTISERA

Antisera		Type of agglutination	Final titers: In tube test, With boiled cultures, C 131	EC 501	In tube test, With living cultures, C 131	EC 501	In slide test, With living cultures, C 131	EC 501
Factor	O 126	O	6,400	–	40	–	–	–
	B 16	B	.	.	800	–	18	–*
	H 2	H	.	.	8,000	16,000	25	25
Experimental	No. 344	O	5,120	–	40	–	–	–
		B	.	.	160	–	8	–*
		H	.	.	–	–	–*	–*
	No. 345	O	5,120	–	40	–	–	–
		B	.	.	320	–	10	–*
		H	.	.	–	–	–*	–*
Control	No. 343	O	3,200	–	20	–	–	–
		B	.	.	?	–	?	–*
		H	.	.	5,120	10,240	25	25
	No. 206	O	6,400	–	40	–	–	–
		B	.	.	?	–	?	–*
		H	.	.	10,240	20,480	25	25

NOTE: – = negative in a dilution of 1:20.
–* = negative in a dilution of 1:2.
. = not tested.
? = titer could not be exactly determined.
C 131 = *E. coli* strain with the antigenic structure O126:K71(B16):H2.
EC 501 = *E. coli* strain with the antigenic structure O55:K59(B5):H2.

(EC 501) strains in both tube and slide agglutination tests. At the same time, the test strains were checked for their antigenic properties by the use of the appropriate O, B, and H factor sera (Table II).

Both experimental antisera (Nos. 344 and 345) revealed equally satisfactory B titers in tube and slide agglutination tests, when tested with the living cultures of the homologous strain C 131. They proved to be free of demonstrable amounts of H agglutinins in the very low dilutions (1:20 and 1:2, respectively) of the sera, since they gave no H type reaction with the same antigen. The absence of H2 antibodies in the experimental sera was confirmed by the use of the control strain (EC 501) that possesses the same flagellar antigen (H2), but different somatic antigens (O55:B5) from those of C 131 (O126:B16). It should be emphasized that owing to their fairly high H2 antigenic content, both test strains were agglutinated by the H2 factor serum in relatively high dilutions. On the other hand, significant amounts of H antibodies were to be found in the control antisera (Nos. 343 and 206) as they yielded positive reactions of a pure H type in high dilutions of the sera with both of the test strains. As a result of this, the B titers of the control sera were not clearly determined, since the strong H reactions interfered with and masked the B agglutinations in the lower dilutions of the sera.

Discussion

The results obtained indicate that the four experimental antisera (Nos. 229, 358, 344, and 345) all meet the requirements of *E. coli* OB diagnostic antisera. They have B titers strong enough for demonstrating the presence of homologous B antigens in adequate dilutions of the sera either in tube or in slide agglutination tests. They possess no demonstrable amounts of H agglutinins even in very low dilutions of the sera; consequently, in reading the results of B agglutinations, the possibility of confusing interference by H reactions is eliminated. The O agglutinins of the sera do not agglutinate living bacteria with homologous O antigens in dilutions higher than 1:20 to 1:40, when the antigen suspension consists mostly of good B forms, but they do agglutinate the same bacteria heated at 100° C for 1 hour in relatively high dilutions (1:5120–1:10,240). Thus, the methods herewith presented proved to be applicable for the production of *E. coli* OB diagnostic antisera without any need of serum absorption, even in cases where actively motile strains must be used for the preparation of the immunizing antigen.

There is a striking difference in the B titers of the experimental O125: K70(B15) antisera (No. 229, 1:100 and No. 358, 1:640), although they were produced by the use of the same immunizing antigen. This may be explained by the different periods of immunization. From the onset of the immunization, blood samples were taken from both rabbits at intervals of 3 to 4 days and tested for the presence of B agglutinins. They were demonstrable from the 10th day on in gradually increasing amounts until the 4th day after the fifth injection, when both rabbit test sera revealed a B titer of 1:640. Rabbit No. 358 was bled out the next day, but the immunization procedure was continued on the other rabbit (No. 229) in the hope of obtaining perhaps an even higher B titer. Instead of this, despite two additional injections, the titer dropped gradually to 1:320, then to 1:160. The immunization was discontinued and the rabbit bled out 8 days after the seventh injection. After preservation, the serum showed a final B titer of 1:100. Apparently, to obtain a satisfactory B titer, the immunization process must not be prolonged over a period of about three weeks.

Analysis of the experimental O126:K71(B16) antisera (Nos. 344 and 345) provides a basis for the comparison of the effectiveness of the complex and simplified methods used in the experiments. According to the data obtained, the simplified method, i.e. the use of phenol alone, is at least as effective as the complex one, i.e. production of H-agglutinable "variant" by the use of homologous H antibodies and subsequent preparation of immunizing antigen from the growth of the "variant" on phenolized agar plates. The use of the complex method still may be advisable, however, especially in instances where a particular *E. coli* strain would not respond promptly to the action of the phenol alone as was the case with strain C 130, the H agglutinability of which (1:200 after the fourth passage) could not be reduced further by additional passages on phenolized agar plates without some decrease in the B agglutinability of the strain. A preliminary treatment with homologous H

antibodies, however, rendered the strain C 130 capable of yielding non-H-antigenic growth on phenolized thiotone agar plates without causing any serious damage to the surface antigen of the strain.

All three control antisera (Nos. 307, 343, and 206) produced with the same experimental strains (C 130 and C 131), but by the use of methods different from those employed in our experiments, contain relatively great amounts of H agglutinins, and consequently they cannot be used as OB diagnostic antisera in their present state. The difference between the effectiveness of the experimental and control methods is rather conspicuous, especially if one considers that both experimental strains were in a state of significantly less vigourous motility and lower H agglutinability, at the beginning of the procedure by the use of the control methods, than they were when the experimental methods were employed. Despite this, the H antigenicity of the strains C 130 and C 131 apparently could not be reduced by the control methods to the same extent as it could be by the methods used in our experiments.

In order to prepare satisfactory OB diagnostic antisera from the control sera obtained, the confusing H antibodies must be removed by the serum absorption technique. This is readily accomplished, if adequate strains possessing homologous flagellar, but heterologous somatic antigens, are at the disposal of the serum producer. Such strains, however, may be unavailable. Besides, the use of serum absorption has additional disadvantages. It is a time-consuming procedure, and may cause a significant decrease in the B titer as a consequence of (sometimes repeated) dilutions of the sera. As the B titer is usually not too high in most of the OB antisera prepared, any further reduction of the titer is undesirable.

There is no need of using the serum absorption technique, if aflagellated strains can be employed for the preparation of OB suspensions. When aflagellated strains are unavailable, methods resulting in artificial reproduction of "variants" with lost or insignificant H antigenicity might ensure the same purpose. The results of the experiments herewith presented prove the possibility of producing such "variants" from actively motile strains.

The experimental data clearly indicate that the homologous H antibodies and the phenol are to be regarded as specific factors in producing non-H-antigenic "variants". It should be emphasized that, actually, we did not produce true variants or mutants, as the motility and H agglutinability (hence, probably, also the H antigenicity) of all our "variants" were restored after several passages on ordinary media containing no specific factors, which fully correspond to the findings of other authors (1, 10). This fact indicates that the process of H variation in our experiments took place in a form of modification and not in that of mutation. Nevertheless, the modifying effect of the specific factors involved still proved to be of practical value in producing satisfactory OB diagnostic antisera.

In this paper no details were given on the mechanism of the H variation in question. Results of studies made on this particular part will be reported in a subsequent publication.

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A TOXIN-INACTIVATING SUBSTANCE (NOXIVERSIN) FROM PENICILLIUM CYANEO-FULVUM (BIOURGE)[1]

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Abstract

A wide range of bacterial toxins are inactivated by a substance extracted from cultures of a strain of *Penicillium cyaneo-fulvum* (Biourge) and named here "Noxiversin". This activity is distinct and separate from an antibiotic produced by the same mold and may be concentrated by fractional precipitation. The albumen fraction of culture filtrates includes all the toxin-neutralizing activity and removal of toxic impurities by precipitation with copper sulphate finally yields a dry stable preparation which is some 10 times more potent than the crude albumen concentrate.

The neutralizing activity has been shown against staphylococcus alpha and beta toxins; streptolysins "S" and "O"; the haemolysins of *Clostridium perfringens* (lecithinase C), *C. septicum*, *C. histolyticum*, and *C. tetani;* tetanus neurotoxin, pertussis toxin, and diphtheria toxin.

Noxiversin exerts its effect quickly and directly on the toxin molecule, destroying toxicity but leaving antigenicity unimpaired. It is thus an effective toxoiding agent. Noxiversin is thermolabile, its activity being destroyed at 60° C in 30 minutes. Preliminary characterization by chemical analysis, Tiselius electrophoresis, and ultracentrifugation methods indicates the presence of two major components in the semipurified product: a homogenous carbohydrate fraction and a heterogeneous protein fraction. Toxin-neutralizing activity resides in the protein fraction.

Introduction

A mold was isolated in 1947 from one of six Löwenstein slopes inoculated with sputum which produced abundant growth of *Mycobacterium tuberculosis* only on the other five. Tested on blood agar the mold showed marked antibiotic activity but with a known penicillin resistant strain of *Staphylococcus aureus* it showed a wide zone of inhibition of haemolysis without interference with growth. Subcultures from the non-haemolytic area onto fresh medium showed normal haemolysis. This mold was identified by Dr. G. A. deVries (Centraalbureau voor Schimmelcultures, Baarn, Nederland) as *Penicillium cyaneo-fulvum* (Biourge).*

Preliminary experiments with filtrates of broth cultures of the mold showed definite and rapid toxin-inhibiting activity, independent of antibiotic activity, against staphylococcus alpha and beta haemolysins in vitro, against the

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*This culture is deposited as *P. cyaneo-fulvum* (Biourge) McGill strain, in The Commonwealth Mycological Institute, Ferry Lane, Kew, England, as No. I.M.I. 58138; in the American Type Culture Collection, 2029 M Street NW, Washington, D.C., as No. 11923; and in Le Musée National d'Histoire Naturelle, Laboratoire de Cryptogamie, 12 rue de Buffon, Paris V.

necrotizing staphylococcus toxin intradermally in rabbits, against tetanus toxin, and, with the help of Dr. Louis Greenberg, against diphtheria toxin and less marked against pertussis toxin. It was shown that this active material is precipitated with the albumen fraction of the mold culture filtrate, that it is not dialyzable, that it is destroyed at 60° C in 1 hour, that it has no antibiotic properties, and that it is not interfered with by "Clarase" (Takamine diastase).

A number of toxin-neutralizing substances present in or isolated from culture filtrates of microorganisms have been described in the literature. Thus Metchnikoff in 1897 (11) and Nicolle in 1907 (15) demonstrated the ability of culture filtrates of *Bacillus subtilis* to inactivate the toxins of *Corynebacterium diphtheriae*, *Clostridium tetani*, *Clostridium chauvei*, those of other bacteria, and certain of the snake venoms. Ramon and Richou (17, 18, 19, 20, 21), Smolems (24), and Richou and Gerbaux (22) have shown that culture filtrates of *B. subtilis*, *Actinomyces griseus*, and *Penicillium notatum* are able to destroy in vitro the haemolytic, lethal, and dermonecrotic effects of staphylococcus and of various other toxins, with contact at 37° C for three or more hours. The principle involved was precipitated out of solution in an active form with sodium sulphate and was found to be separate and distinct from the antibiotics produced by the same organisms. The active principle so obtained was thermolabile and non-dialyzable. It inactivated the toxins and was said to destroy the immunizing and combining power. Del Vecchio *et al.* (7) precipitated a substance from culture filtrates of *P. notatum* by one-third saturation with ammonium sulphate which neutralized staphylococcus and diphtheria toxins and which did not destroy the flocculating power of diphtheria toxin. Cavalli (5) showed in filtrates of *A. griseus* a heat-labile substance which neutralized staphylococcus toxins and Nélis (12) isolated bacteria from fresh waters of which the filtrates neutralized staphylococcus and diphtheria toxins. Reports on the toxin-neutralizing activity of the conventional antibiotics are conflicting. Although impure preparations of penicillin might have some toxin-neutralizing activity the capacity to inactivate toxins disappears with increasing purification of the antibiotic. Clavacin is said not to affect the toxicity of staphylococcus toxins (4) whereas in vitro it is claimed to inactivate tetanus toxin (13, 14, 16).

Noxiversin from *P. cyaneo-fulvum* differs in one or more aspects from each of these descriptions in the literature. Because of the speed of its action on a diversity of toxins and because the loss of toxicity it induced resulted in an immunizing toxoid (anatoxin), the product of this mold seemed to be of more than ordinary interest.

Procedure

Maintenance of the Penicillium Culture

Cultures of the McGill strain of *P. cyaneo-fulvum* have been maintained in the deep freeze at −21° C, since its isolation in 1947, with no loss in essential

characters and certainly no loss in its ability to produce a potent toxin-neutralizing derivative. The mold may be propagated on a variety of solid media; maximum growth is obtained on malt extract agar and it is upon this medium that stock and seed inoculum cultures are maintained. The mold grows most luxuriantly at 20° C and under ordinary atmospheric conditions, with a maximum yield of mycelium in 7 days. Stock cultures are stored at 4° to 6° C for periods of not longer than 3 weeks after which time some variation in color and other minor characters is likely to occur; for this reason, subcultures are made at 3-week intervals.

Preparation of the Toxin-Inactivating Material

Various media have been tested for production of the toxin-inactivating factor. The highest yields have been obtained in a 1% glucose – beef infusion – peptone broth. Lower yields are obtained with plain peptone broth and malt extract media. Raulin's medium and a Czapex-Dox medium, modified by Del Vecchio (6, 7) for the production of a toxin-neutralizing substance from *P. notatum*, have failed to yield a toxin-inactivating substance with our species of *Penicillium*.

Lots of 250 ml glucose broth contained in 1000-ml Roux bottles are inoculated with a heavy glucose broth suspension of mycelium harvested from a stock culture. The cultures are allowed to incubate at room temperature and, without shaking, to form a heavy surface mat of mycelium. The pH of the medium falls from 7.2 at the time of inoculation to approximately 5.0 by the 5th day, after which it rises again to reach pH 7.5 by the 7th day. At this time the cultures are transferred to the cold room to await further processing.

The mycelium is removed by filtration through Whatman No. 1 paper followed by filtration through a Zigmondy–Sartorius filter membrane to remove spores. Although the active principle is strongly adsorbed by filter paper, once the paper is well soaked with filtrate no subsequent appreciable loss occurs. No loss is incurred in membrane filtration. The pH of the filtrate is adjusted to 7.0 and ammonium sulphate is added to a concentration of 48% of saturation (36.9 g per 100 ml) at room temperature to precipitate the globulin fraction. The globulin fraction, which is dark gray in color, is removed by membrane filtration since a Seitz filter has been found to adsorb a high portion of the active principle. The globulin precipitate which collects on the membrane is, if washed, almost completely devoid of toxin-neutralizing activity. The clear filtrate contains the active albumen fraction and this is precipitated by adding further ammonium sulphate, 40 g per 100 ml, to yield full saturation at room temperature. This albumen fraction precipitate is harvested by membrane filtration, and while still wet is dissolved in 0.85% sodium chloride solution (10 ml for each 100 ml of original culture filtrate). The solution is dialyzed against saline for 48 hours at 5° C and −6 mm of mercury pressure. At the end of the dialysis period, the dry weight of the material is 130 mg/ml ± 20 mg. In earlier experiments the

albumen fraction was stored and used as the final product for tests of toxin-neutralizing activity. To this end the material was lyophylized and stored *in vacuo* at 4° to 10° C, although there is evidence that the crude albumen fraction in the lyophylized form is quite stable even at room temperature. The further purification by copper sulphate precipitation of impurities was not developed until late in the course of the investigation. To the dialyzed albumen solution at room temperature, copper sulphate is added to a concentration of 1.0%. A voluminous orange precipitate forms, complete in 30 minutes, and the precipitate may be removed by centrifugation at 2800 to 3000 r.p.m. for 20 minutes. The clear, light-green supernatant fluid is decanted and dialyzed against 0.85% sodium chloride solution for 48 hours at 5° C and −6 mm of mercury. The final yield of purified material is 212.8 mg for each 100 ml of crude culture filtrate. The dialyzed fluid is lyophylized and the powder stored *in vacuo* in sealed glass ampoules at 5° C and 1 mg of the final product has a neutralizing ability equal to that of 9.8 mg of the dry crude albumen fraction, i.e. it is some 10 times more potent on a unit weight basis. The copper sulphate procedure removes virtually all of the inactive impurities, some of which are toxic to animals; the final product is relatively atoxic for the animal body and the very slight reaction on subcutaneous injection in man seems attributable to the small amount of copper salt remaining.

Results

Since the copper sulphate procedure for precipitation of impurities from the crude albumen fraction was devised late in the course of the investigation, many of the tests reported in this paper concern the toxin-neutralizing activity of the crude albumen fraction rather than that of the more highly purified material now available. Some comparisons are given of the potency of the latter product, in terms of neutralizing activity for a given toxin, with that of the crude albumen fraction.

The activity of the mold derivatives has been tested in vitro against a variety of bacterial toxins, including *Staphylococcus aureus* alpha toxin (haemolytic, dermonecrotic, and lethal effects), *S. aureus* beta haemolysin, *C. perfringens* alpha toxin (lecithinase C), *C. tetani* neurotoxin, *C. septicum* haemolysin, streptolysin "O", streptolysin "S", and the toxins of *Corynebacterium diphtheriae* and *Haemophilus pertussis.*

TABLE I

ANTIBIOTIC ACTIVITY OF WHOLE CULTURE FILTRATES OF *P. cyaneo-fulvum*

++++	*Streptococcus pyogenes* (Lancefield A), *Staphylococcus aureus, Neisseria gonorrhoeae, Neisseria meningitidis, Bacillus subtilis*
+++	*Streptococcus salivarius, Diplococcus pneumoniae, Neisseria catarrhalis* and another pharyngeal *Neisseria, Clostridium tetani, Clostridium perfringens*
++	*Haemophilus influenzae*
0	*Klebsiella pneumoniae* (Type A), *Escherichia coli, Aerobacter aerogenes, Proteus morganii, Pseudomonas aeruginosa, Streptococcus faecalis, Candida albicans*

NOTE: The measurement of activity is not quantitative but ++++ was a very wide zone of inhibition of growth and 0 indicates no interference at all.

TABLE II

STRAPHYLOCOCCUS ALPHA HAEMOLYSIN INACTIVATION TITER OF SEMIPURIFIED MOLD DERIVATIVE

	Toxin activity control										
*Dilution of haemolysin in 1 ml	1/1	1/1	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256
	1 ml of 2% rabbit RBC's to each tube and incubated 1 hour at 37° C										
Haemolysis	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
	Toxin inactivation test										
Staphylococcus alpha haemolysin, ml	1/1	1/1	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256
Dilution of mold derivative, 150 mg in 1 ml	1/4	1/2	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
	After incubated 1/2 hour at 37° C 0.5 ml 5% rabbit RBC's to each tube and incubated 1 hour at 37° C										
Haemolysis	++	0	0	0	0	0	0	0	0	0	0

*The titer of the toxin was 1600 Burnet Units/ml. Therefore 1 ml of the mold derivative solution neurtalizes 3200 Burnet Units of staphylococcus alpha toxin, i.e. 150 mg of the lyophylized mold derivative neutralizes 3200 Burnet Units.

An extensive survey of the quantitative in vitro effect of the mold derivatives has been made on staphylococcus alpha toxin. Indeed, neutralization of this particular toxin has served as the yardstick for assay of potency of the mold products throughout the course of the investigation. Staphylococcus alpha toxin was prepared from the Wood 46 strain of *S. aureus*. The toxin, depending on the batch, has ranged in potency from 1600 to 3200 Burnet Units (B.U.) per ml of culture filtrate. The ability of the mold product to neutralize staphylococcus alpha haemolysin is demonstrated by mixing 1-ml volume dilutions of a saline solution of Noxiversin (150 mg/ml) with 1-ml volume dilutions of staphylococcus alpha haemolysin (1:1 to 1:256) and, after 30 minutes at 37° C in a water bath, 1 ml of a 2% suspension of rabbit erythrocytes is added to each tube. Haemolysis of the rabbit corpuscles is read after 1 hour at 37° C. A typical titration is shown in Table II. By incubation of the mold derivative – toxin mixtures for varying intervals of time before the addition of the red cells it has been shown that maximum inactivation of the toxin occurs in from 10 to less than 30 minutes. A period of 30 minutes has been chosen as the standard incubation period. Some 20 controlled experiments were performed to determine the neutralizing ability of crude unconcentrated culture filtrates and of albumen fraction concentrates of *P. cyaneo-fulvum* cultures against staphylococcus alpha haemolysin. The results are presented in Tables III and IV. A rather marked variation

TABLE III

VARIATION IN POTENCY OF SUCCESSIVE BATCHES OF CRUDE CULTURE FILTRATE OF *P. cyaneo-fulvum* IN INACTIVATING STAPHYLOCOCCUS ALPHA TOXIN, IN EQUIVALENT OF BURNET UNITS PER ML OF FILTRATE

Batch	1	2	3	4	5	6	7	8	9	10
B.U./ml	16	40	100	120	60	100	100	400	100	100
Batch	11	12	13	14	15	16	17	18	19	20
B.U./ml	60	60	60	800	80	100	100	120	1600	100

TABLE IV

POTENCY OF 150 MG DRY OF DIFFERENT BATCHES OF ALBUMEN FRACTION OF MOLD DERIVATIVE TO INACTIVATE STAPHYLOCOCCUS ALPHA TOXIN (IN BURNET UNITS) AND ITS EQUIVALENCE TO PROVISIONAL INTERNATIONAL UNITS OF STANDARD STAPHYLOCOCCUS ANTITOXIN (P.I.U.)

Batch	B.U. toxin inactivated	P.I.U. equivalence	Batch	B.U. toxin inactivated	P.I.U. equivalence
1	3200	—	11	3200	18
2	1600	—	12	3200	13
3	1600	—	13	12800	52
4	12800	—	14	3200	16
5	1600		15	3200	13
6	1600	—	16	1600	9
7	1600	—	17	1600	9
8	1600	—	18	3200	16
9	3200	17	19	3200	13
10	3200	13	20	3200	16

in yield, which cannot be explained at the present time, is encountered in the potency of the whole culture filtrates, as is seen in Table III. Depending on the batch, 1 ml of filtrate may neutralize from 16 to 1600 Burnet Units of staphylococcus alpha haemolysin. It is obvious, however, that these values are extreme, only occasionally met with, and 1 ml of most batches neutralizes 100 to 400 Burnet Units, with an over-all average of 200 Burnet Units. Rather more consistent results are obtained with the albumen fraction concentrates. In a series of 20 batches with the exception of two lots which have shown exceptionally high neutralizing values (12,800 Burnet Units neutralized by 150 mg of the albumen fraction) the neutralizing capacity of 150 mg of the albumen fraction has ranged from 1600 to 3200 Burnet Units. By direct comparison employing the method of Hartley and Smith (10) and using a standard antitoxin with, in each instance, the same batch of toxin, it was shown that 1 ml of the albumen concentrate (containing 150 mg on a dry weight basis) is the equivalent in neutralizing capacity of from 9 to 52 Provisional International Units (P.I.U.) of staphylococcus anti-alpha haemolysin (Table IV), varying with the batch and averaging out to 17 P.I.U. of antitoxin. Thus, approximately 9 mg of the dry albumen fraction represents the equivalent in neutralizing capacity of 1 P.I.U. of staphylococcal anti-alpha haemolysin.

The inactivation of the dermonecrotizing effect of *S. aureus* toxin by the mold albumen concentrate shows a close correlation with the inactivation of haemolysis by the toxin. The staphylococcus toxin used in the experiments contained 1500 Burnet Units and 13 LH doses per ml as measured against standard alpha antitoxin. The minimal dermonecrotic reacting dose (Lr) of this batch of toxin as measured in rabbits was 0.5 ml when the toxin was mixed with 1 P.I.U. of antitoxin. By serial titration it was shown that the minimal dermonecrotic reacting dose of the toxin was 0.5 ml when mixed with 1/32 ml, or 4.68 mg dry weight, of the albumen concentrate. Thus 1 ml of this albumen concentrate, containing 150 mg dry weight, has approximately the same neutralizing potency as 16 P.I.U. of antitoxin; this result shows a close correlation with the inactivation of the haemolytic activity of the toxin.

In one experiment 1.5 ml (225 mg dry weight) of the albumen concentrate neutralized in vitro 2.5 rabbit lethal doses of staphylococcus alpha toxin (the equivalent in this instance of 3500 Burnet Units); this has not been repeated. It has been shown, however, that the albumen concentrate when mixed with toxin in amounts just sufficient to neutralize haemolytic activity renders the toxin non-lethal for mice whereas controls receiving untreated toxin in the same amounts die within 1 hour.

The precipitation of impurities from the crude albumen fraction with copper sulphate has resulted in a marked increase in potency of the final product. The supernatant fluid following precipitation of impurities with copper sulphate contains the toxin-neutralizing derivative of *P. cyaneo-fulvum* in virtually full yield; 1 ml of the supernatant fluid with a dry weight of 15.2

mg has essentially the same neutralizing capacity as 1 ml of the crude albumen concentrate with a dry weight of 150 mg. Table VI shows a representative series of batches in which 15.2 mg of the final purified product neutralizes from 1000 to 4850 Burnet Units of staphylococcus alpha haemolysin. The purified material has proved on the whole some 10 times more potent on a weight basis than is the parent crude albumen fraction from which it is derived.

In dealing with toxins other than staphylococcus alpha toxin, for which a well-defined unit of haemolytic activity exists, a standard haemolytic unit had to be adopted. There is at present no standard unit by which comparative results may be expressed. For this reason the principle of the Burnet Unit has been used in this investigation to express the potency of all bacterial haemolysins and it is referred to simply as the haemolytic unit. One haemolytic unit is that amount of toxin, which causes 50% haemolysis of 1 ml of a 2% suspension of red blood cells, contained in a total volume of 2 ml in 1 hour at 37° C. The haemolysin inactivation tests were performed in the same manner as is described above for staphylococcus alpha haemolysin.

The results obtained with a variety of bacterial haemolysins are presented in Table V. The neutralizing capacity of the albumen concentrate is, in each instance, expressed as the number of haemolytic units neutralized by the standard test dose of 150 mg of the crude albumen concentrate.

S. aureus beta haemolysin prepared and provided by Rublee (23) contained 256 haemolytic units per ml and 150 mg of a crude albumen concentrate of *P. cyaneo-fulvum* of average potency neutralized quite consistently in four tests 128 haemolytic units of beta lysin (Table V). The serum-extractable lysin of *Streptococcus pyogenes* was prepared by culturing the organism in 100 ml of Todd's broth (25) for 16 hours at 37° C. The cells were harvested by centrifugation, washed twice with saline, and the lysin was extracted by shaking the cell sediment in 10 ml of sterile horse serum for 2 hours at room temperature and the extracted cell bodies were removed by centrifugation; the supernatant fluid possessed a titer of 1024 haemolytic units per ml. As is seen in Table V, 150 mg of the crude albumen concentrate neutralized some 512 haemolytic units of streptolysin. Streptolysin "O", the oxygen-labile haemolysin of *Streptococcus pyogenes*, was prepared by growing the organism in 100 ml of Todd's broth for 16 hours at 37° C followed by removal of the cells by centrifugation and the supernatant fluid contained 16 haemolytic units per ml. In one experiment it was shown that 150 mg of the albumen concentrate was required to inactivate eight haemolytic units, a relatively low value compared to the neutralizing capacity of the mold derivative for other bacterial haemolysins.

Clostridium perfringens alpha toxin (lecithinase C) in a partially purified preparation was used and contained 400 intraperitoneal M.L.D.'s for 20-g mice and 160 haemolytic units per ml. With 150 mg of a crude albumen concentrate of *P. cyaneo-fulvum* of average potency, 32 to 160 haemolytic units were neutralized in five experiments with a mean of 134 units (Table V).

TABLE V

NEUTRALIZATION OF BACTERIAL HAEMOLYSINS BY THE ALBUMEN CONCENTRATE OF *P. cyaneo-fulvum* CULTURES

Haemolysin source	Experiments	No. of haemolytic units neutralized by 150 mg of crude albumen concentrate	Mean
Staphylococcal alpha lysin	20	(See Table III)	3520
Staphylococcal beta lysin	1	128	
	2	128	
	3	128	128
	4	128	
Streptolysin "S"	1	512	512
	2	512	
	3	512	
Streptolysin "O"	1	8	
C. perfringens alpha toxin (lecithinase C)	1	160	
	2	160	
	3	32	134.4
	4	160	
	5	160	
C. septicum haemolysin	1	20	
	2	64	
	3	20	28.8
	4	20	
	5	20	
C. histolyticum haemolysin	1	64	
	2	64	64
	3	64	
C. tetani haemolysin	1	64	
	2	80	
	3	80	76.8
	4	80	
	5	80	

C. perfringens alpha toxin is a single component, lecithinase C, which, in addition to its ability to bring about haemolysis of red blood cells, causes necrosis of tissue cells and is lethal for experimental animals when injected in suitable concentrations. On this basis it may be assumed that with neutralization of haemolytic activity by the mold derivative there is a corresponding neutralization of dermonecrotizing and lethal effects, similar to that shown experimentally for staphylococcus alpha toxin.

A *C. septicum* toxin in the form of a whole culture filtrate prepared according to the method of Adams and Hendee (1) contained 20 haemolytic units per ml. From 20 to 64 haemolytic units were neutralized by the standard 150 mg test amount of albumen concentrate, with a mean of 28 units for five experiments (Table V). In addition to the neutralizing capacity of the crude albumen concentrate the effect of purified Noxiversin has been tested on *C. septicum* haemolysin, and, as is shown in Table VI, 1 mg of the purified product neutralizes some 12 to 20 haemolytic units. Thus it may be calculated that 150 mg of the purified material might neutralize from 1800 to 3000 units of *C. septicum* haemolysin.

TABLE VI

THE NEUTRALIZATION OF BACTERIAL HAEMOLYSINS BY PURIFIED NOXIVERSIN

Haemolysin	Experiments	No. of haemolytic units neutralized by 1 mg of purified Noxiversin	No. of haemolytic units neutralized by 150 mg of purified Noxiversin (by calculation)	No. of haemolytic units neutralized by 150 mg of crude albumen fraction (by test)
Staphyloccus alpha lysin	1	124	18,734	3520
	2	65	9,860	(Mean of 20 tests)
	3	210	31,552	
	4	293	44,000	
C. septicum haemolysin	1	12	1,800	28.8
	2	20	3,000	(Mean of 5 tests)
	3	20	3,000	
	4	20	3,000	
	5	20	3,000	

Whole culture filtrates of *C. septicum* prepared according to the methods of Adams and Hendee (1) and Bernheimer (3) have failed in our hands to produce dermonecrosis in rabbits in any volume amenable to intradermal injection. A potent semipurified dermonecrotizing toxin of *C. septicum* was obtained, however, by the method of Bengston (2). The minimal dermonecrotic dose as determined by titration in the skin of the rabbit was found to be 1.09 mg contained in 0.2 ml of saline. The least amount of purified Noxiversin capable of neutralizing one minimal dermonecrotic dose was 0.36 mg.

C. histolyticum toxin in the form of a whole culture filtrate, prepared by the same method as was employed for the preparation of *C. septicum* toxin, contained 32 haemolytic units per ml; and 75 mg of the albumen concentrate was required to neutralize the haemolytic activity of 1 ml of filtrate in each of three experiments. Thus it is calculated that 150 mg of the crude mold derivative neutralizes 64 units of *C. histolyticum* haemolysin (Table V). The haemolysin of *C. tetani* was prepared by the method adapted to the preparation of the haemolysins of *C. septicum* and *C. histolyticum*. A whole culture filtrate possessed 40 haemolytic units per ml and 150 mg of the albumen concentrate neutralized some 64 to 80 haemolytic units in five experiments with a mean of 76 units (Table V).

The ability of Noxiversin to neutralize toxic activities other than haemolytic has already been noted in the close correlation seen between neutralization of the haemolytic effect and the dermonecrotic and lethal properties of staphylococcus alpha toxin. It has also been noted that, in addition to its capacity to neutralize the haemolytic effect of whole culture filtrates of *C. septicum*, the mold derivative neutralizes the dermonecrotizing activity of semipurified *C. septicum* toxin. It is commonly held that the haemolytic, dermonecrotic, and lethal effects of staphylococcus alpha toxin are properties of one molecule exerting different effects on different substrates. Whether or not this is true of *C. septicum* toxin is not clear. It will be noted that

whole culture filtrates of *C. septicum* possessed no dermonecrotic activity, although they possessed haemolytic activity, and that special media and procedures had to be employed for the production of a dermonecrotizing toxin.

As additional evidence that Noxiversin neutralizes toxins other than haemolysins, the effect of the mold derivative on tetanus neurotoxin is presented. It has been shown that Noxiversin neutralizes tetanolysin. However, tetanus haemolysin and tetanus neurotoxin are distinct and separate substances. The neurotoxin used in the experiments was a filtrate of a Robertson's meat culture which had been incubated at 37° C for 4 days. In tests for neutralization activity the toxin was incubated in admixture with varying concentrations of purified Noxiversin at 37° C for 1 hour. The concentration of Noxiversin in the reaction mixtures is expressed in milligrams of the mold derivative per minimal lethal dose (M.L.D.) of toxin for 20-g mice (intraperitoneal). Control samples of toxin in volumes of saline equal to the volumes of Noxiversin solution were incubated at 37° C for 1 hour, since it has been noted that some spontaneous deterioration of tetanus neurotoxin occurs at this temperature within 1 hour. Test and control mixtures were evaluated by intraperitoneal injection in 20-g white mice in terms of typical symptoms of tetanus toxaemia and mortality rates at 4 and 10 days. The results of experiments, involving three batches of Noxiversin, are shown in Table VII. The data indicate a definite neutralizing capacity of Noxiversin for tetanus neurotoxin, shown in the 4-day end points although at 10 days the mortality rate of mice for the weaker Noxiversin begins to approach that of the controls. It will be noted that with the most powerful batch of Noxiversin (batch 3) 0.11 mg completely neutralized 1 M.L.D. of tetanus toxin, so that all of 10 mice remained alive and well at 10 days whereas the control toxin killed 9 of 10 animals with typical paralysis within 4 days. In another experiment where LD_{50} rather than

TABLE VII

THE IN VITRO EFFECT OF PURIFIED NOXIVERSIN ON TETANUS TOXIN. MORTALITY OF 20-G MICE INOCULATED WITH MIXTURE OF 1 M.L.D. TOXIN WITH VARYING NOXIVERSIN; INCUBATED 1 HOUR AT 37° C BEFORE INJECTION

Noxiversin* batch No. (mg dry Noxiversin)	Observation time (days)	Mortality					Mortality control: 1 M.L.D. toxin in saline at 37° C for 1 hour
		0.9	0.45	0.12	0.11	0.09	
1	4	1/10	2/10			5/10	10/10
	10	7/10	9/10			8/10	10/10
2	4			4/10			8/10
	10			9/10			9/10
3	4				0/10		9/10
	10				0/10		9/10

*Noxiversin potency of 1 mg dry against staphylococcus alpha haemolysin in Burnet Units. Batch 1 neutralizes 75 B.U.; batch 2 neutralizes 11 B.U.; batch 3 neutralizes 166 B.U.

M.L.D. values of toxin were employed it was shown that 0.04 mg of purified Noxiversin completely neutralized 1 LD_{50} of tetanus neurotoxin with respect to both 4- and 10-day end points. By calculation it may be determined that 1 mg of purified Noxiversin neutralizes 25 LD_{50} of standard tetanus toxin. By direct comparison with the same toxin it was shown that 1 International Unit of tetanus antitoxin neutralized 2048 LD_{50} of the tetanus toxin. Thus 1 mg of Noxiversin has the equivalent neutralizing capacity of 0.012 units of antitoxin.

Experiments performed early in the course of the investigation indicated that crude culture filtrates of *P. cyaneo-fulvum* exerted a neutralizing effect on the dermonecrotizing activity of pertussis toxin, but this has not been carried further.

Although we have been unable to devise an in vitro test for the action of Noxiversin on diphtheria toxin, there is evidence, to be presented in a subsequent publication, that the mold derivatives when administered to guinea pigs infected with *C. diphtheriae* lowers the mortality rate, and this despite a complete lack of any antibiotic activity of Noxiversin on the organism.

Some Properties of the Toxin-Neutralizing Derivative of P. cyaneo-fulvum

1. Effect of temperature.—The toxin-neutralizing factor is thermolabile, having essentially the same range of heat lability as protein. The active principle as it is found in the albumen concentrate shows some 75% inactivation at 60° C in 30 minutes in a solution of 150 mg of dry crude albumen concentrate per ml in saline and it is completely inactivated at 75° C in 30 minutes (Table VIII).

2. The effect of filtration.—Seitz filtration completely removes, presumably by adsorption, the active principle from solutions of the albumen concentrate and from the more highly purified preparations. Why this is so is not clear since it is not appreciably absorbed from solution by animal charcoal, activated charcoal, or kaolin. The Sartorius membrane filters do not absorb the active principle and allow complete recovery in the filtrate.

3. The effect of normal animal sera on the toxin-neutralizing factor.—The toxin-neutralizing activity of albumen concentrates and of the more highly purified Noxiversin preparations is inactivated by normal animal blood serum. Tests were performed by incubating 0.5 mg dry weight per ml, an amount sufficient to neutralize 800 Burnet Units of staphylococcus alpha lysin, with 0.5 ml of serum, at 37° C for 30 minutes. It was shown that the serum of

TABLE VIII

EFFECT OF TEMPERATURE ON THE TOXIN-NEUTRALIZING FACTOR OF *P. cyaneo-fulvum*
(Crude albumen fraction, 150 mg/ml)

Temperature, ° C	30	40	50	60	70	80	90	100
Time, minutes	30	30	30	30	30	30	30	30
No. of B.U. of staph. alpha lysin neutralized by 150 mg of albumen concentrate	1600	1600	1600	1200	0	0	0	0
% inactivation	0	0	0	75	100	100	100	100

TABLE IX

THE EFFECT OF CRYSTALLINE SERUM ALBUMEN AND ALPHA, BETA, AND GAMMA GLOBULINS ON THE TOXIN-NEUTRALIZING FACTOR

Serum constituent	Potency of toxin-neutralizing factor before treatment: B.U. of staph. alpha lysin/150 mg	Potency of toxin-neutralizing factor after treatment: B.U. of staph. alpha lysin/150 mg	% inactivation
Albumen	1600	0	100
Alpha globulin	1600	1600	0
Beta globulin	1600	1600	0
Gamma globulin	1600	1600	0

the guinea pig, rabbit, horse, and human, whether untreated, heated at 60° C for 30 minutes, or Seitz-filtered, brought about complete inactivation of the toxin-neutralizing principle. Purified Noxiversin does not differ from the albumen concentrate in this respect. In order to determine the constituent of animal serum responsible for this, the effect of crystalline serum albumen and crystalline alpha, beta, and gamma globulins were tested. It is obvious from the data presented in Table IX that the ability of serum to inactivate the toxin-neutralizing factor resides entirely in the serum albumen fraction.

Toxicity of the Mold Derivative for Experimental Animals

Since work to be reported in a later publication has been directed to the therapeutic possibilities of Noxiversin, an investigation of the toxicity of the product for animal tissues has been made. Rabbits and guinea pigs are insusceptible to quite large doses of the crude concentrated mold derivative. Rabbits withstand, with no untoward effect, a single intravenous dose of 1500 mg of the albumen concentrate, an amount sufficient to neutralize at least 10 intravenous rabbit lethal doses of staphylococcal alpha toxin. Doses of 2400 mg administered intravenously in 300 mg doses, at regular intervals, over a period of 3 weeks produce no symptoms in the rabbit. No minimum lethal dose for the rabbit has been established with the available amount of material. Guinea pigs weighing 400 g are completely unaffected by 900 mg of the crude albumen concentrate in a single intraperitoneal dose; however, 1200 mg is lethal. Mice of 20 g are insusceptible to 6 mg intraperitoneally of the albumen concentrate, but 15 mg results in symptoms of shock with death in about 1 hour. The antihistaminic neo-antergan (Poulenc) protects mice against this effect as has been noted by others (22) in the case of toxin-neutralizing substances derived from other molds.

Purified Noxiversin, although lethal for mice in a dose of 30 mg, is completely free of toxicity in a dose of 15 mg. Whereas 15 mg of the crude albumen concentrate is lethal for mice and inactivates in vitro only 320 Burnet Units of staphylococcus alpha toxin, 15 mg of purified Noxiversin is non-toxic for mice and inactivates 3200 Burnet Units.

Noxiversin as a Toxoiding Agent

Preliminary studies have been made in an attempt to determine the mode of action of the mold derivative. It has been shown that washed rabbit

red cells if suspended in a concentrated solution of the albumen concentrate (75 mg of mold derivative per 0.5 ml of a 5% red cell suspension) incubated for 37° C for 1 hour and then washed free of the mold derivative, are as susceptible to haemolysis by staphylococcus alpha haemolysin as are normal rabbit red blood cells. This indicates that the toxin-neutralizing factor reacts directly with the toxin rather than by combining with the toxin-susceptible tissue and thus blocking the action of the toxin.

Richou and Gerbaux (22) have described a toxin-neutralizing substance isolated from *Penicillium notatum* which, in reacting with staphylococcal toxin, destroys its antigenicity and combining power. Del Vecchio *et al.* (6, 7), on the other hand, investigating a similar substance state that there is no loss in combining power. Our product has been proved to be an efficient toxoiding agent. Staphylococcus alpha toxin with a titer of 1600 Burnet Units per ml was completely inactivated by incubation with 75 mg of dry albumen concentrate of *P. cyaneo-fulvum* per ml of toxin at 37° C for 1 hour. Critical tests were performed to ensure that no active haemolysin remained. A rabbit weighing 4 kg was immunized with the mixture according to an immunization regime proposed by Dolman (8) for the usual formalized toxoid. Following immunization the animal's serum contained 1 P.I.U. of staphylococcal anti-alpha haemolysin per ml of serum and it withstood, without symptoms, an intravenous challenge dose of 1800 Burnet Units of the toxin (approximately 1.2 lethal doses) whereas three non-immunized control animals of the same weight died within 1 hour following the injection of a similar dose of toxin.

Tests for the combining power of staphylococcal alpha haemolysin, completely inactivated with the concentrated mold albumen fraction, were performed according to the method of Dolman and Kitching (9). In one test a batch of toxin containing 41 Lf units was completely detoxified, by incubation for 1 hour with the mold derivative, and showed no loss in combining power with its equivalent of antitoxin. In another experiment toxin so treated showed a drop from 50 Lf to 20 Lf, a loss not greater than that frequently encountered in the conventional process of formol toxoiding. A rabbit, treated with 2400 mg of the albumen concentrate administered in 300-mg doses over a period of 3 weeks, did not develop any detectable precipitating antibody for any component of the crude toxin-neutralizing preparation. It is not feasible, except of involved techniques, to test such sera for antibody capable of neutralizing the biological activity of the toxin-neutralizing derivatives, since the albumen fraction of normal serum exerts a potent effect in this respect.

Characterization of Noxiversin

Preliminary studies have been made to determine the chemical nature of the toxin-neutralizing particle. The purified product contains 33% of protein. Qualitative tests are strongly positive for carbohydrate and protein and negative for free lipids. Preliminary studies of Noxiversin by paper and Tiselius electrophoresis and by ultracentrifugation methods indicate

the presence of two separable fractions. One, a major component of relatively low mobility, is homogeneous, carbohydrate in nature, and has a sedimentation constant of approximately 5.0 Svedberg Units. The carbohydrate fraction is devoid of toxin-neutralizing activity. A second fast-moving component, which has proved to be protein in nature, is heterogeneous showing some three to four peaks all having approximately the same mobility. The over-all sedimentation constant of the protein complex has ranged in a number of tests between 0.58 and 1.13 Svedberg Units. It has been shown that the toxin-neutralizing activity of Noxiversin preparations is contained in the heterogeneous protein complex. Further resolution of the protein fraction has not been achieved at this time.

Summary

The McGill strain of *P. cyaneo-fulvum* (Biourge) produces in suitable media a substance capable of inactivating in vitro a wide range of bacterial toxins. Of various media tested glucose – beef infusion broth has given the highest yields of the toxin-neutralizing principle. Although the *Penicillium* strain does produce an antibiotic with an antibacterial range closely related to that of penicillin (Table I), it has been shown that the antibiotic and the detoxifying factor are distinct substances. The detoxifying agent may be obtained in a concentrated form and free of the antibiotic by fractional precipitation of the culture filtrate with ammonium sulphate, eliminating the inactive globulin fraction and retaining the active albumen fraction precipitated by full saturation. After reconstitution in saline, and dialysis, further inactive toxic material is precipitated by 1% copper sulphate and the dialyzed lyophilized supernatant yields a stable powder approximately ten times more active than the crude albumen precipitate. The final product, called Noxiversin, inactivates various different bacterial toxins. It readily inactivates in vitro the haemolytic, dermonecrotizing, and lethal properties of staphylococcus alpha toxin. By comparison, 150 mg of the crude albumen fraction has a neutralizing capacity equivalent to that of between 9 and 52 Provisional International Units of staphylococcus alpha antitoxin. On this basis it may be calculated that 150 mg of purified Noxiversin is the equivalent of some 90 to 520 Provisional International Units of antitoxin. The albumen concentrate also readily inactivates in vitro a variety of bacterial haemolysins such as staphylococcal beta lysin, streptolysins "S" and "O", *C. perfringens* alpha toxin (lecithinase C), *C. septicum* haemolysin, *C. histolyticum* haemolysin, and *C. tetani* haemolysin. The mold derivative tested in the form of the more highly purified Noxiversin inactivates the necrotizing factor secreted by *C. septicum*. It is not clear whether or not this is identical with the haemolysin, but since the dermonecrotizing factor required special techniques and media for its preparation, not required for the production of the haemolysin, the two may be distinct. As further evidence that the activity of the mold derivative is not limited to haemolysins, data are presented which indicate the in vitro activity of purified Noxiversin on tetanus

neurotoxin, which is quite distinct from the haemolysin secreted by the same organism. Preliminary experiments also indicate activity of the mold product against pertussis toxin and diphtheria toxin.

Experiments directed to ascertain the mode of action of Noxiversin indicate clearly that the active principle acts directly upon the toxin rather than upon the substrate. In this respect Noxiversin has been shown to be an effective toxoiding agent, in that it completely detoxifies staphylococcus alpha toxin after 1 hour at 37° C, without marked destruction of immunizing and combining power.

The active principle is thermolabile, being largely inactivated at a temperature of 60° C and completely inactivated at 70° C in 30 minutes. It is inactivated by serum—whether untreated, heated at 60° C, or Seitz-filtered—of the guinea pig, rabbit, horse, sheep, and human; the albumen fraction of serum has been shown to be entirely responsible for this effect. Tiselius electrophoresis and ultracentrifugation studies of semipurified Noxiversin show the presence of two major components: one, a homogeneous carbohydrate fraction of low mobility with a sedimentation constant of 5 Svedberg units, and the other, a fast-moving heterogeneous protein fraction showing four peaks, all having approximately the same mobility and an average sedimentation constant of from 0.58 to 1.13 Svedberg units. The toxin-neutralizing activity resides in the protein fraction.

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THE INFLUENCE OF CERTAIN FACTORS ON THE IN VITRO PRODUCTION OF ERGOT ALKALOIDS BY CLAVICEPS PURPUREA (FR.) TUL.[1]

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Abstract

Eighteen of 41 isolates of *Claviceps purpurea* produced a detectable quantity of the ergot alkaloids under standard conditions. Large differences in yield indicated that strain differences existed. There was no correlation, however, between the capacity to produce alkaloids and either the species of host from which the culture was isolated or the pigmentation of the culture.

The time of incubation and the composition of the medium were important in determining whether natural light stimulated or inhibited the synthesis or accumulation of ergot alkaloids.

Commercial galactose, which was contaminated with glucose and lactose, was the most efficient carbon source examined, but the rate of synthesis was markedly reduced if the galactose was purified before use. The activity of commercial galactose could be replaced by a mixture of pure galactose and glucose but not by a mixture of galactose and lactose. A strong interaction existed between the concentration of commercial galactose and phosphate. Ammonium succinate, soybean meal, urea, and yeast extract were the most efficient nitrogen sources examined. L-Tryptophane could not serve as a nitrogen source for growth but when added as a supplement influenced alkaloid production. Succinic but not citric, tartaric, phosphoric, acetic, or sulphuric acid could be used successfully to adjust the initial pH of the production medium. The replacement culture technique did not shorten the incubation time required for alkaloid production.

Production of alkaloid, growth, and consumption of carbon and nitrogen proceeded at a steady rate until the 43rd day when the rate decreased markedly. Organic nitrogen but not polysaccharide was secreted into the medium.

The total yield and the rate of alkaloid synthesis was influenced by the size of the inoculum and by the nature of the agent used to suspend the inoculum, but not by the number of times the inoculum was washed. The rate of production was very slow if the inoculum source was not homogenized.

Introduction

The production of small amounts of ergot alkaloids by cultures of *Claviceps purpurea* (Fr.) Tul. was reported in a previous publication (12). The yields obtained in this study were not satisfactory for an investigation of the biosynthesis of the alkaloids. Additional work has therefore been carried out on the culture conditions used (2) in order to find out which factors were essential for alkaloid production, and whether certain modifications might lead to an increase in yield. The results of these studies are reported in this paper.

Materials and Methods

The Culture

Forty-one cultures of *Claviceps purpurea* were screened using the conditions and medium of Abe (2) in order to examine the influence of strain type on alkaloid production and to select a high-producing culture for more detailed

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study. Culture PRL 1578 was chosen. It produced mainly ergosine and ergosinine, with smaller amounts of ergometrine, ergometrinine, ergotamine, ergocornine, ergokryptine, ergokryptinine, and three unknown alkaloids. Tryptophane but not tryptamine was produced when the culture was grown on the medium No. 10-A-galactose. This culture requires biotin for growth as do all isolates of *Claviceps purpurea* so far examined (11).

The Inoculum

Unless stated otherwise, the inoculum for each experiment was started individually from an agar slant culture kept under sterile mineral oil as follows: A fragment was transferred to 70 ml of medium No. II (12). The resulting culture was incubated 5 to 7 days at 22° to 25° C on a shaking machine revolving at 150 r.p.m. It was then homogenized in a Waring blendor and 1.0 ml added to a second flask of this medium. Following incubation for 2 or 3 days, the secondary culture was diluted with physiological saline, homogenized, and washed twice with saline using centrifugation. The washed mycelium was suspended in 20 ml saline, homogenized 3 seconds, and made to 100 ml with saline. One milliliter was added to each experimental flask of medium within 30 minutes of preparation. Three 10-ml aliquots of the suspension were removed from each inoculum preparation to determine the dry weight equivalent of the inoculum. The dry weight of 1.0 ml suspension ranged from 0.9 to 2.7 mg; the value aimed at being 1 mg/ml. An improved inoculum preparation was developed late in the course of these studies and it is described under the appropriate heading.

The Prototype Medium

Since Abe's medium (2)(medium No. 1) contains tap water and hence is not completely defined, a modification of his medium was used. The prototype medium (No. 10-A) was prepared in the following manner:

Solution A:

Deionized mannitol (or equivalent in carbon)	50 g
Distilled water	200 ml

The solution was autoclaved 15 minutes at 18 lb pressure.

Solution B:

Ammonium succinate (or equivalent in nitrogen)	8.0 g
KH_2PO_4	1.0 g
$MgSO_4.7H_2O$	0.3 g
$FeSO_4.7H_2O$	5.0 mg
$CaCl_2.2H_2O$	5.95 mg
$ZnSO_4.7H_2O$	4.40 mg
$MnSO_4.H_2O$	2.75 mg
NaCl	2.55 mg
$CuCl_2.2H_2O$	2.269mg
$(NH_4)_6Mo_7O_{24}.7H_2O$	1.82 mg
Succinic acid	to pH 5.2
Distilled water	800 ml

Forty milliliters of solution B were added to each 250-ml Erlenmeyer production flask. The flasks were autoclaved 15 minutes at 18 lb pressure and, after cooling, 10 ml of solution A and 1 ml of solution C were added to each flask. The final pH was 5.2.

Solution C:

Biotin	25 μg
Distilled water	100 ml

Each carbon source was routinely deionized by treatment with a mixture of Dowex 1-X8 (CO_3^-) and Amberlite IR-120-X8 (H^+), since it was found that commercial mannitol contained sufficient lead to suppress yield. It was subsequently found, however, that deionization of galactose did not result in significantly higher yields. Labile compounds such as tryptophane, urea, gluconate, glucuronate, biotin, and ammonium salts were sterilized by filtration through an ultrafine sintered glass filter.

Culture Conditions

Cultures were grown in 51 ml medium contained in 250-ml Erlenmeyer flasks which had been wrapped in black friction tape, then in aluminum foil. A loose, shallow aluminum foil cap made by molding foil over the bottom of a 200-ml centrifuge bottle was placed over the cotton plug. Each cotton plug was cut to a predetermined size and used only once. The cultures were incubated in a stationary position at 22° to 25° C in a forced draft incubator. Humidity was not controlled.

Analysis of Cultures

Ergot alkaloid.—The whole culture was homogenized, freeze-dried, rewetted with 1.0 ml of 10% aqueous ammonia, and extracted with 50 ml of ether. The ethereal solution was back extracted with 0.2 *N* sulphuric acid, and the aqueous phase treated with van Urk reagent according to the method of Allport and Cocking (1). The optical density of the solution was determined at 550 mμ and the amount of alkaloid present calculated by comparison with the color yield from solutions containing known amounts of ergometrine maleate.

Dry weight.—Growth was measured as described previously (11).

Sugar.—Reducing sugar and total carbohydrate in the culture filtrate, which had been corrected for evaporation, was determined by the Somogyi and Anthrone methods respectively as described by Neish (6).

Nitrogen.—Residual ammonium nitrogen in the filtrate was determined by titrating the ammonia released from boiling an aliquot of the filtrate with magnesium oxide. The ammonia was trapped in 2% boric acid containing methyl red and bromcresol green. This procedure did not release ammonia from solutions of peptone. Organic nitrogen in the filtrate was estimated as the difference between Kjeldahl and ammonium nitrogen.

Analysis of Results

The yield of alkaloid was recorded as the arithmetic mean and standard error of the analysis of three replicate cultures. Means of similar magnitude

were compared by the statistical analyses referred to previously (10). A level of $P < .05$ was taken to indicate evidence for the existence of a significant difference. Where an interaction was suspected, sequential experiments employing factorial analyses were employed. The selection of intervals for the succeeding experiments was aided by plotting the results as contour yields (8) and by concentrating on the areas of steepest ascent.

Cultures were routinely harvested after 35 and 50 days' growth. Since the time for maximal yield varied from experiment to experiment, the reported yields are not necessarily optimal and they are to be compared only with the control of the same experiment. An examination of controls from six early experiments revealed, for example, that the average coefficient of variation for replicates was 14%, while the coefficient of variation of all 18 values was 44%

Results and Discussion

Strain Selection

A comparison of the ability of 41 cultures of *Claviceps purpurea* to produce alkaloid on medium No. 1 (2) is given in Table I. Eighteen cultures produced detectable quantities, using blue to green colors with van Urk reagent and a

TABLE I

SCREEN OF 41 CULTURES OF *Claviceps purpurea*
(50 days)

PRL No.	Host	µg total alkaloid/liter	Van Urk color	Mycelium color
1574	*Lolium perenne*	280	Blue-green	Red
1571	*Agrostis alba*	<75	Pink	Cream
1586	*Calamagrostis canadensis*	490	Blue-green	Brown
1572	*Bromus inermis*	<75	Pink	Cream
1595	*Avena fatua*	<75	Pink	Cream
1589	*Secale cereale*	80	Blue	Cream
1587	*Poa pratensis*	280	Blue	Tan
1565	*Glyceria borealis*	1700	Blue-green	Cream
1591	*Agropyron smithii*	80	Blue	Cream
1564	*Bromus ciliatus*	380	Blue	Gray
1582	*Phleum pratense*	90	Blue	Red
1576	*Arrhenatherum elatius*	280	Blue	Cream
1597	*Agropyron cristatum*	<75	Pink	Tan
1566	*Agropyron albicans*	400	Blue	Cream and gray mottled
1569	*Secale cereale* (albino sclerotium)	280	Blue-brown	Cream
1577	*Dactylis glomerata*	<75	Pink	Cream
1593	*Bromus inermis*	<75	Pink	Gray and brown mottled
1568	*Stipa viridula*	<75	Pink	Gray and cream mottled
1570	*Agropyron dasystachum*	1390	Blue	Dark brown
1585	*Bromus ciliatus*	510	Blue	Pink
1580	*Bromus inermis*	<75	Yellow	Red
1583	*Calamovilfa longifolia*	530	Blue	Brown
1567	*Triticum vulgare*	<75	Yellow	Cream
1578	*Phleum pratense*	3540	Blue	Red
1588	*Agrostis exarata*	300	Blue	Cream
1594	*Calamagrostis canadensis*	<75	Yellow	Cream
1590	*Agropyron* sp.	<75	Yellow	Cream
1596	*Calamagrostis inexpansa*	690	Blue	Brown
1573	*Avena sativa*	<75	Pink	Cream
1592	*Calamagrostis neglecta*	340	Blue	Buff
1584	*Bromus pumpellianus*	130	Blue	Gray
1579	*Spartina gracilis*	200	Blue	Cream
1598	*Secale cereale*	<75	Deep red	Yellow
1652	*Phleum pratense*	<75	Colorless	Brown
1651	*Secale cereale*	<75	Yellow	Yellow-brown
530	Unknown	<75	Gray	Cream
1654	*Bromus* sp.	<75	Colorless	Yellow
1655	*Glyceria borealis*	<75	Colorless	Yellow
1649	Unknown	230	Blue	Buff
1653	*Festuca elatior*	<75	Yellow	Cream
1664	*Secale cereale*	<75	Colorless	Cream

value of at least 100 μg/liter as the test criteria. No correlation existed between the capacity to produce ergot alkaloids and either the species of host from which the culture was isolated or the pigmentation of the culture. The results indicate clearly, however, the importance of strain on the amount of alkaloid produced. Simultaneously, several other fungi were examined. Isolates of *Aspergillus flavipes*, *Coniothyrium fuckelii*, *Ustilago bulatii*, *Ustilago trebouxii*, and *Ustilago maydis* did not produce alkaloids under these conditions. *Penicillium roqueforti* PRL 1463 gave a positive van Urk color test and paper chromatography (12) of the alkaline ether extract showed this to be due to a number of substances three of which were identical in R_f value with elymoclavine, penniclavine, and agroclavine. An unidentified species of *Penicillium* did not give a positive test.

Effect of Light on Production

Since the ergot alkaloids are degraded by light, low yields might be caused by exposure of the cultures to light. A direct comparison was made of cultures exposed to natural light and of cultures protected from light by wrapping the flasks in aluminum foil. Exposure to light caused an apparent increase in the rate of synthesis by young cultures grown on medium No. 1 (Table II)

TABLE II

PRL 1578

YIELDS OBTAINED ON TWO MEDIA WHEN EXPOSED TO AND PROTECTED FROM LIGHT

(Milligrams alkaloid/liter culture)

	Medium No. 1		Medium No. 10-A	
Days	Light	Darkness	Light	Darkness
24	0.3±0.01	0.2±0.02	0.1±0.1	0.3±0.08
35	4.1±0.5	2.6±0.4	0.8±0.06	1.1±0.2
50	4.3±0.4	>11.6	4.3±0.7	5.9±0.8
60	6.5±0.7	16.5±0.8	3.2±0.5	4.4±0.5
75*	6.0±0.3	16.0±1.2	4.6±0.2	5.1±0.5

*By 75 days almost all liquid had evaporated but the mycelial pad was still intact.

but later it inhibited either synthesis or accumulation. Natural light does not affect cultures grown on medium No. 10-A. However, if the pH of the latter medium is adjusted with sulphuric acid, alkaloid is not produced (Table III), when the culture is exposed to light. There is, then, an interaction between light and sulphuric acid but not between light and succinic acid.

Effect of Carbon Source on Production

Although mannitol and sucrose have been used as carbon sources (2, 3, 4, 9), investigators have not compared the amount of alkaloid produced on various carbon sources. The present studies have, however, shown that the carbon

TABLE III

PRL 1578, MEDIUM No. 10-A

EFFECT OF LIGHT ON PRODUCTION BY CULTURES ADJUSTED WITH SUCCINIC AND SULPHURIC ACIDS

(Milligrams alkaloid/liter)

	Succinic acid			Sulphuric acid	
Days	Light	Comparison*	Darkness	Light	Darkness
26	0.3±0.04	$t = 0.1$	0.3±0.06	None	0.1±0.07
34	2.6±0.6	$t = 0.5$	2.2±0.3	None	0.1±0.06
50	2.1±0.02	$t = 0.9$	1.9±0.2	None	0.4±0.1

*$P > .05$.

TABLE IV

PRL 1578

YIELDS OF ALKALOID FROM CULTURES GROWN ON VARIOUS CARBON SOURCES

(Milligrams/liter)

	Days	
Carbon source*	35	50
Glucose	0.6±0.2	0.8±0.2
Sorbitol	0.2±0.1	0.3±0.03
Mannose	0.3±0.03	0.2±0.01
Mannitol	0.6±0.1	0.5±0.05
Galactose	11.0±0.9	10.8±0.5
Dulcitol	None	None
K-gluconate†	(No growth)	(No growth)
Na-glucuronate†	(No growth)	(No growth)
Galactose	7.3±0.7	18.7±1.6
Sucrose	3.3±0.7	2.4±1.2
Fructose	0.5±0.05	0.3±0.1
Lactose	0.6±0.3	14.3±0.2
Maltose	0.9±0.1	1.9±0.2

*Sugars and alcohols deionized and autoclaved separately. Basal medium No. 10-A.
†Filter-sterilized.

source is an important factor in determining the yield of alkaloids (Table IV) Commercial galactose gave the highest alkaloid yield and hence was used in all subsequent studies (medium No. 10-A-galactose). However, while galactose from two commercial sources gave similar results, galactose purified by passage through a charcoal column and by crystallization from aqueous methanol was inferior (Table V). Growth was also slow on the purified galactose. Paper chromatography showed commercial galactose to contain glucose and lactose and these contaminants were added to pure galactose to determine if they were responsible for the high activity of commercial galactose. The activity of commercial galactose could be replaced by a mixture of pure galactose and glucose but not by a mixture of pure galactose and commercial lactose, and it was concluded that commercial galactose was a

TABLE V

PRL 1578

EVIDENCE THAT THE ACTIVE CONTAMINANT OF COMMERCIAL GALACTOSE IS GLUCOSE AND NOT LACTOSE*

(Milligrams/liter)

Expt.	Sugars	Days			
		30	35	40	50
1	Commercial galactose		28.3±2.7	21.9±0.8	19.4±1.1
	Purified galactose		2.5±0.2	8.9±0.9	16.2±1.0
	Commercial lactose		<0.5	<0.5	<0.5
	Purified galactose + lactose (9+1)		6.3±1.1	11.5±0.8	20.1±0.5
2	Commercial galactose	40.3±2.7	39.1±3.0	48.1±3.1	
	Purified galactose	0.8±0.2	–	–	
	Glucose	–	1.5±0.4	1.86±0.1	
	Purified galactose + glucose (9+1)	38.2±2.1	60.1±4.4	52.3±5.1	
	Purified galactose + glucose (9.5+0.5)	52.2±1.8	63.0±2.3	55.9±2.6	

*Paper chromatography showed the presence of glucose and lactose in this lot of commercial galactose.

superior carbon source because it was a mixture of galactose and glucose (Table V). The possibility is being considered that galactose utilization requires adaptation and that glucose serves as a primer.

Effect of Nitrogen Source on Production

Ammonium succinate is used as the nitrogen source in Abe's medium whereas Stoll has reported high yields using asparagine in combination with sucrose as the nitrogen source. PRL 1555 grows poorly on a combination of mannitol and asparagine and produces little alkaloid whether mannitol or glucose is used as the carbon source. PRL 1578, on the other hand, grows well on a combination of mannitol and asparagine. These data suggests a possible strain specificity with respect to nitrogen utilization. With PRL 1578 ammonium succinate, soybean meal, filter-sterilized urea, and yeast extract were the most efficient nitrogen sources of those examined (Table VI). Only nitrate failed to support production.

L-Tryptophane would not support growth when used as the sole nitrogen source. When added to medium 10-A-galactose at a concentration of 500 mg/liter, the yield was doubled. Lesser amounts were not stimulatory, nor did ammonium succinate at equivalent nitrogen concentration alter the yield. Paper chromatographs (12) of extracts from supplemented and control cultures revealed that the tryptophane-treated cultures contained agroclavine, penniclavine, and possibly a little tryptamine, while the controls did not.

TABLE VI

PRL 1578

YIELDS OF ALKALOIDS FROM CULTURES GROWN ON VARIOUS NITROGEN SOURCES IN COMBINATION WITH GALACTOSE

(Milligrams alkaloid/liter culture)

Nitrogen source	35 days*	pH	50 days
Ammonium succinate	9.4±1.6	5.8	22.4±0.1
Yeast extract	3.9±1.0	6.1	12.9±0.6
Asparagine	0.6±0.08	6.8	2.5±0.5
Bacto peptone	0.7±0.02	6.0	7.1±0.04
Soybean meal	9.3±0.7	6.3	42.0±0.4
Ammonium succinate	9.7±0.4	5.5	17.6±1.0
Potassium nitrate	None	7.5	None
Ammonium phosphate	4.2±0.7	3.2	4.4±1.0
Glutamic acid	5.7±0.6	7.5	4.0±0.7
Urea	None	5.6	None
Urea (filter-sterilized)	7.4±0.4	6.5	13.9±0.8
Tryptophane (filter-sterilized)	(No growth)	6.1	(No growth)
Ammonium succinate	16.1±0.7	5.6	10.7±0.5
Ammonium succinate + 500 mg tryptophane per liter†	23.1±0.5	5.8	21.5±1.7

*23-day-old cultures contained little or no alkaloid.
†Added at 24 hours.

Thus, while tryptophane may not be a desirable supplement from the point of view of increasing the yield of "natural" alkaloids, it does influence the course of indole metabolism.

Effect of Medium Composition on the Type of Alkaloids Formed

Since the composition of the production medium affected the quantity of alkaloid formed, it was necessary to establish also whether or not such modifications readily affected the types of alkaloid synthesized. The alkaloids of cultures grown on media No. 1, No. 10-A, and No. 10-A, but containing soybean meal as the nitrogen source, were identified by paper chromatography. No qualitative differences were detected and it was concluded that the type of alkaloid formed is not readily influenced by changes in the composition of the medium

Effect of Organic Acids on Production

Abe *et al.* (3) found that maximum yields were obtained when the initial pH of the medium was adjusted to 5.2–5.4. Succinic acid was used. In order to determine if this was the most efficient acid, succinic acid was compared with citric, tartaric, phosphoric, and acetic acid in medium 10-A when nitrogen was supplied as the ammonium salt of each acid or in a duplicate experiment when nitrogen was supplied as ammonium hydroxide. In both experiments the pH was adjusted to 5.2 with each acid. A high yield was obtained only on the medium containing succinic acid (Table VII). Succinic acid is still present in cultures after 50 days. Succinic acid is a necessary constituent of the

TABLE VII

PRL 1578

EFFECT OF ACIDS USED TO ADJUST pH OF MEDIUM ON PRODUCTION

(Milligrams alkaloid/liter culture)

	Days				
	35		50		60
Acid	Alkaloid	pH	Alkaloid	pH	Alkaloid
Succinic	17.0±1.1	7.3	9.4±0.2	8.0	7.9±0.2
Citric	0.7±0.03	5.4	0.7±0.02	7.6	0.6±0.04
Tartaric	None	4.2	None	4.0	None
Phosphoric	None	5.5	None	5.5	None
Acetic	(No growth)	5.2	(No growth)	5.2	(No growth)

NOTE: Medium No. 10-A-galactose with ammonium nitrogen added as filter-sterilized ammonium salts of the corresponding acids. Comparable results were obtained when using NH_4OH as the nitrogen source.

medium but it is possible that its beneficial effect may be due only to a regulation of pH change.

Production by Stationary and Replacement Cultures

Abe stated that the time for maximal yield was reduced if the replacement culture technique was used. Since this technique might offer certain advantages to the production of alkaloids, its effect on production was ascertained. Production on medium No. 10-A-galactose by stationary and replacement cutlures is shown in Fig. 1. Replacement with 50 ml of fresh 10-A-galactose medium was made on the 20th day. The replacement technique did not shorten the time required for maximal production when the total age of the culture was considered. Similar results were obtained when replacement was made on the 15th day.

Proportion of Galactose to Potassium Phosphate

It was not known whether the proportions of galactose to phosphate in medium No. 10-A were optimal, or whether an interaction between the two was possible. This information was sought through factorial designs involving three levels of each variable. The sequential method was used. Two experiments were completed before it was learned that the contaminants of galactose were responsible for the high rate of production (Table VIII). The results have been included, however, because they do provide some information concerning optimal proportions and interaction.

Firstly, there is a strong interaction between the carbon source and phosphate. The concentrations of 65 to 80 g of commercial galactose and 0.25 g potassium acid phosphate are near optimal. Secondly, there are two trends leading toward increased yields: one involving low concentartions of phosphate and one was the highest concentration. This second trend may be due to a secondary effect such as regulation of pH.

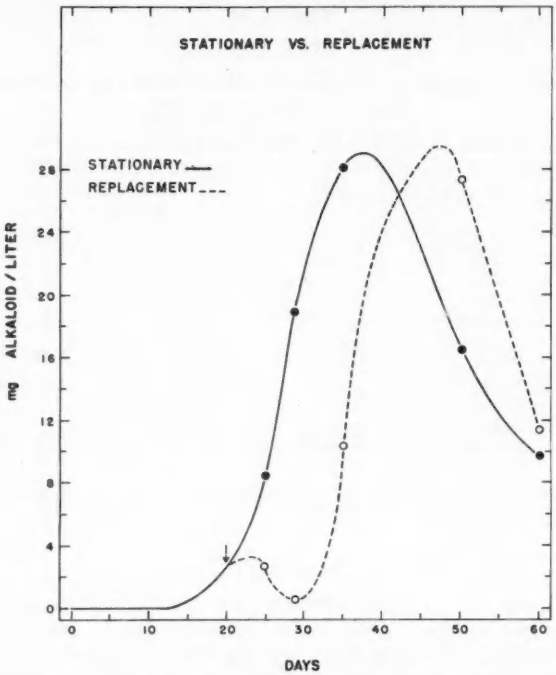

FIG. 1. Alkaloidal content of stationary and replacement cultures. The cultures were grown on medium No. 10-A-galactose. On the 20th day replacement cultures were prepared by decanting off the growth medium and replacing it with fresh medium.

TABLE VIII

PRL 1578

INTERACTION BETWEEN GALACTOSE AND PHOSPHATE

(Milligrams alkaloid/liter, 35 days)

Galactose, g/liter	KH_2PO_4, g/liter				
	Factorial No. 2			Factorial No. 1	
	0.1	0.15	0.25	1.0	3.0
35	–	–	19.0±3.6	16.6±2.3	27.7±1.8
50	–	–	49.4±1.8	30.1±1.1	40.7±2.5
65	13.4±0.6	59.5±0.9	76.0±4.2	24.3±2.9	39.6±1.3
			74.8±4.2	–	
85	3.5±0.1	39.7±3.5	80.3±5.2	–	–
100	3.6±0.1	11.9±0.3	59.8±0.7	–	–

NOTE: The data for 35 and 50 days of factorial No. 1 indicated a vector of increase in yield toward increasing galactose and decreasing phosphate. This determined the design of factorial No. 2. The dashes represent combinations not tested.

*Proportion of medium No. 10-A-galactose.

The production medium was modified to contain 65 g commercial galactose and 0.25 g KH_2PO_4 per liter medium (medium No. 10-B).

The Course of Alkaloid Synthesis and Growth

The fact that alkaloid synthesis took place only after incubation periods of at least 20 days suggested that synthesis followed rather than accompanied growth. Abe concluded that synthesis occurred only when rate of growth was slow. Growth inhibitors such as alpha-naphthalene acetic acid increased yields somewhat. A time-course study of production by 1578 on the medium modified by using 65 g galactose and 0.25 g potassium acid phosphate per liter (medium No. 10-B) was undertaken. The cultures were started with 1.0 ml water-suspended inoculum and although the yields were not as high as expected, the data are believed to be typical of the producing culture.

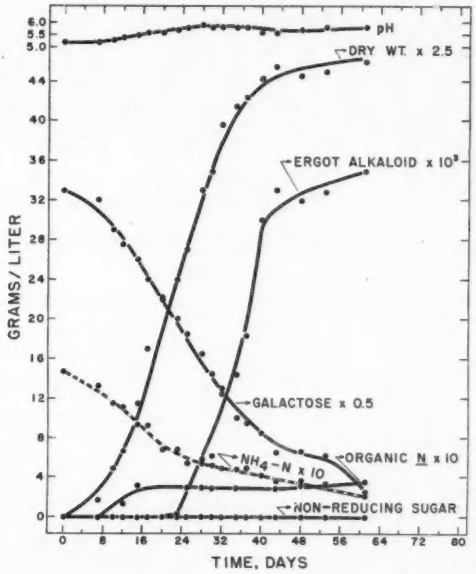

FIG. 2. The course of alkaloid synthesis and growth. The alkaloid values represent alkaloid in mycelium and filtrate. Organic nitrogen represents the difference between Kjeldahl and ammonia nitrogen in the filtrate after being brought back to the original volume. There was essentially no difference between carbohydrate values obtained from the Anthrone and Somogyi tests.

The production of alkaloid, growth, carbon, and nitrogen utilization all slowed up markedly on approximately the forty-third day (Fig. 2). Alkaloid synthesis, then, does accompany growth, and it appears that production does not begin until the mycelial pad has been formed. Under the conditions used, alkaloid degradation was not noticeable. The extent of degradation appears to vary with the composition of the medium or with some factor whose identity is not known (compare Figs. 1 and 2, and Tables II, V, and XI).

It is interesting to note that Michener and Snell (5) found that alkaloid added to cultures of *Claviceps purpurea* was degraded more rapidly if a reducing sugar was present than if not.

Lysis of the mycelial pad did not occur. By the 61st day the dried mycelium consisted of 3.4% nitrogen. Essentially no carbohydrate but some organic nitrogen was released into the medium. The quantity of organic nitrogen in the filtrate did not increase appreciably after the 15th day. Presumably some of the organic nitrogen exists as ergot alkaloids, since it has been demonstrated that appreciable alkaloid can be released into the medium (2).

Homogenizing the Inoculum

One of the factors considered responsible for the slow rate of synthesis was injury to the inoculum resulting from homogenizing the inoculum source for 3 seconds. A comparison of yields by cultures started with equivalent quantities of homogenized and unhomogenized mycelia revealed, however, that alkaloid synthesis and growth were actually accelerated by the treatment (Table IX). The homogenizing procedure was found by Savage and Vander Brook (7) to permit the use of very small inocula in penicillium production but the rate of synthesis was slowed somewhat.

TABLE IX

PRL 1578

EFFECTS OF HOMOGENIZING THE INOCULUM SOURCE ON YIELDS

(Milligrams alkaloid/liter)

	Days		
Treatment*	24	30	35
Not homogenized	Nil	1.7±0.4	16.6±0.6
Homogenized	16.6±1.7	39.9±2.3	–

*One secondary flask culture was the source of inoculum. Half of the production flasks were inoculated with 1 ml of culture. The remainder was then homogenized 3 seconds and 1.0 ml added to each of the remaining flasks.

Washing the Inoculum

If the synthesis of alkaloid was influenced by substances in the medium used to grow the inoculum, then variations in the washing procedure might be responsible for the variations in yield from experiment to experiment. Synthesis was found not to be influenced by the number of washings of the inoculum source (Table X). The washing procedure was maintained, however, to prevent possible interaction between constituents of the growth medium and production medium.

Effect of the Quantity of Inoculum on Production

The standard procedure for preparing the inoculum involved the use of physiological saline. Previous studies (10) indicated that the performance of *Claviceps purpurea* was influenced by the size of inoculum and it was

TABLE X

PRL 1578, MEDIUM NO. 10-A-GALACTOSE

EFFECT OF WASHING INOCULUM ON PRODUCTION

(Milligrams alkaloid/liter culture)

Saline washes of homogenized secondary culture	Days	
	35	50
None	24.1±1.5	8.9±0.5
1	26.0±0.7	13.6±1.8
2	24.5±1.0	14.2±1.5
3	28.0±0.9	17.6±0.9

NOTE: There was no significant difference among the values for 35 days.

concluded that the smallest acceptable inoculum should be employed so that the performance of a culture would not be unduly influenced by substances carried into the production medium with the inoculum. Therefore the effect of various quantities of the standard mycelial suspension on production was considered first (Table XI (A)). A large quantity of mycelial suspension suppressed yield and this was found, by direct tests, to be due to the saline thus introduced into the production medium (Table XII). When the mycelial suspension was made up in distilled water, the yield increased, to a limit,

TABLE XI

PRL 1578

EFFECT OF VARIOUS QUANTITIES OF STANDARD MYCELIAL SUSPENSION ON PRODUCTION

(Milligrams alkaloid/liter)

Inoculum size	Days*		
	35	49	60
	(A) Suspended in physiological saline†		
0.1	7.5±0.9	7.9±0.5	4.8±1.0
0.5	12.7±0.6	7.3±0.4	3.3±0.8
1.0	8.4±0.4	5.9±0.08	3.1±0.6
5.0	4.1±0.8	2.3±0.3	2.2±0.4
10.0	1.5±0.09	0.9±0.2	0.8±0.2
	(B) Suspended in distilled water‡		
0.1	17.3±0.1	23.1±1.5	11.8±2.0
0.5	22.8±6.0	27.8±1.8	20.0±0.5
1.0	28.1±1.5	27.8±4.6	23.5±0.8
5.0	30.1±1.4	24.4±1.4	20.6§
10.0	39.8±0.8	37.2±1.3	23.5±1.4

*Values of 20-day-old cultures were very small.
†Analysis: (A) Interaction between inoculum size and time significant. At 35 days yields were largest on 0.5 ml inoculum; at 49 days 0.1 and 0.5 ml were equivalent and best.
‡Analysis: (B) At 35 days increase in yield with increase in inoculum is significant.
§Single value.

TABLE XII

PRL 1578, MEDIUM No. 10-B

SUPPRESSION OF YIELD BY SODIUM CHLORIDE PRESENT IN A LARGE INOCULUM

(Milligrams alkaloid/liter culture)

Treatment	Days	
	35	50
Small inoculum (1 ml) in saline	30.9±2.2	33.0±1.7
Small inoculum plus 9 ml saline	5.7±1.1	2.7±0.4
Large inoculum (10 ml) in saline	8.0±1.2	3.4±0.2
Large inoculum in water*	25.6±1.2	22.1±4.3

*The saline suspension was centrifuged, decanted, and made to volume with distilled water.

with increase in inoculum size (Table XI (B)) and this was due to the quantity of mycelium, not the quantity of water (Table XIII). There is an upper limit, however, and the use of all of the mycelium from a secondary growth culture to inoculate a single flask results in strongly suppressed yields.

TABLE XIII

PRL 1578, MEDIUM No. 10-B

EFFECT OF VARIOUS QUANTITIES OF MYCELIUM OF PRODUCTION WHEN SUSPENDED IN 10 ML OF WATER

(Milligrams alkaloid/liter)

Dry weight (mg) equivalent of inoculum	Days		
	30	35	44
24*	12.6±2.4	38.1±3.9	40.8±8.4
13	10.7±1.2	26.0±3.0	41.4±1.8
9	8.7±0.9	21.0±2.6	21.4±1.0
0.9†	1.7±0.5	10.3±2.4	12.3±2.0
0.45	2.1±0.3	7.5±0.06	9.6±1.4

*Production very low when using an inoculum equivalent to 413 mg (entire secondary culture).
†Equivalent in weight to standard suspension.

A direct comparison of water and saline as suspending agents confirmed the superiority of water (Table XIV). While a large inoculum suspended in water would be suitable for production of the ergot alkaloids, it would not be as suitable for fundamental studies because of the carry-over of substances in the mycelium. For the latter type of studies, an inoculum approximately equivalent to 1.0–2.0 ml of the standard water-suspended inoculum source would appear to be suitable from the point of view of obtaining high yields with the smallest quantity of inoculum.

TABLE XIV

PRL 1578, MEDIUM No. 10-B

A DIRECT COMPARISON OF SALINE AND WATER AS SUSPENDING AGENTS FOR THE INOCULUM

(Milligrams alkaloid/liter)

Agent	Days		
	30	35	45
Saline*			
0.5 ml	19.4±2.2	49.8±5.4	50.5±3.7
1.0 ml	26.2±2.3	42.4±0.7	48.4±1.4
10.0 ml	<0.5	<0.5	1.2±0.04
Water*			
0.5 ml	23.6±2.7	47.8±3.8	53.5±1.4
1.0 ml	32.2±1.1	50.2±0.7	54.4±0.9
10.0 ml	36.7±4.0	56.8±4.6	55.8†

*1.0 ml of each inoculum suspension was equivalent to 2.7 mg dry mycelium.
†Single value.
Analyses: (*a*) Comparison of water and saline: (1). 0.5 ml inocula were equivalent; (2) 1.0 and 10.0 ml water inocula were superior.
(*b*) Comparison of time with concentrations of water inocula; (1) on the 30th day the size of the inoculum was critical; (2) the inoculum size had no effect on yield by the 35th and 45th day.

The slope of yield on inoculum size is quite steep in the low range and this fact was probably responsible for much of the variation that existed among experiments. Another source of variation exists, however, which affects total yield (compare Tables XIII and XIV and Fig. 2 with Table VIII) and its identity is being sought.

Acknowledgments

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THE BIOSYNTHESIS OF SOME AMINO ACIDS IN PENICILLIUM DIGITATUM[1]

DONALD J. REED,[2] VERNON H. CHELDELIN, AND CHIH H. WANG

Abstract

The pathways of biosynthesis of alanine, serine, glycine, aspartic acid, and glutamic acid in *Penicillium digitatum* have been studied by means of tracer techniques, using glucose-2-C^{14} and glucose-6-C^{14} as carbon sources. Alanine appears to be derived directly from pyruvate formed in the glycolytic degradation of glucose. Serine is synthesized from glycine, which is in turn derived mainly from a C_2 fragment that originates in the C_2–C_3 cleavage of pentose, a product of phosphogluconate decarboxylation. The biosynthesis of aspartic acid in this organism may involve several pathways. Glutamic acid appears to be synthesized from glucose intermediates via the conventional reactions of the TCA cycle.

Introduction

Previous papers from this laboratory (3, 7, 9) have reported the utilization of C^{14}-labelled CO_2, acetate, pyruvate, and glucose by intact cells of *Penicillium digitatum*. These have included a quantitative study of the pathways of glucose catabolism in this organism employing the radiorespirometric method of Wang *et al.* (12). The presence of a C_1–C_5 cleavage pathway and glycolysis in conjunction with the tricarboxylic acid cycle (TCA) was indicated. The role played by an "active" C_2 unit thought to be derived from C-2 and C-3 of glucose was demonstrated, which accounted for the observation that the incorporation of C-2 of glucose into the cellular constituents was slightly greater than that of C-6 (7).

In the present work glucose-2-C^{14} and glucose-6-C^{14} have been used as sole carbon sources for growing *P. digitatum* cells in order to trace the origin of the carbon skeletons in the biosynthesis of glycine, serine, alanine, aspartic acid, and glutamic acid.

Material and Methods

Cells, grown in a manner described previously (3), were harvested, washed, and suspended in a medium deprived of carbon sources, containing ammonium ions and other inorganic salts. Two 1-liter growth flasks were connected to the manifold of a macroscale radiorespirometer (12) for a time-course study of metabolic pathways. To each flask was added a cell suspension containing 1.5 g (dry weight basis) of cells and the organisms were starved for 90 to 120 minutes by vigorous agitation with CO_2-free air (400–600 ml per minute). Glucose-2-C^{14} was then added to one of the flasks (750 mg, containing

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50 μc radioactivity). An identical amount of glucose-6-C^{14} was administered to the other flask. Aeration was maintained at the same rate for an additional 7 hours.

The cells in each flask were then harvested by filtration, washed with distilled water, with 80% alcohol, and finally with a small amount of ether, then dried *in vacuo* over P_2O_5 in preparation for subsequent processing. The washings were evaporated *in vacuo* and the residual solubles were added to the cells prior to hydrolysis.

Isolation of Amino Acids

The cells were hydrolyzed by 0.05 *N* HCl in the presence of Dowex-50 resin (H^+ form) in a manner similar to that described by Paulson *et al.* (5). In preliminary experiments, it was found that nearly all the hydrolyzable polysaccharides in the mycelia could be isolated from the supernatant fraction as monosaccharide when the mycelia were refluxed gently with the resin at 100° C for 10 to 12 hours and the resin removed by centrifugation. After the removal of the supernatant liquid, the hydrolysis of the cellular protein was completed. The resulting amino acids were then eluted from the Dowex-50 resin with 1.5 *N* HCl followed by 6 *N* HCl. Basic amino acids, such as arginine and lysine, were recovered in somewhat reduced yields owing to incomplete elution from the resin. The eluted amino acids were freed of excess HCl by repeated evaporation to dryness *in vacuo*.

For the isolation of individual amino acids, the mixed amino acid hydrochlorides were dissolved in a small volume of 0.5 *N* acetic acid and transferred onto a Dowex-1 (8% cross linkage, acetate form) column and fractionally eluted with 9.5 *N* acetic acid according to the procedure of Hirs *et al.* (2). Tyrosine, glutamic acid, and aspartic acid fractions were found to be chromatographically pure by paper chromatography. The glutamic acid fractions were combined, evaporated repeatedly to dryness *in vacuo* to remove the acetic acid, and then diluted with an appropriate amount of carrier L-glutamic acid. The diluted glutamic acid was isolated as the hydrochloride salt. The aspartic acid fractions were treated in a similar manner and the diluted aspartic acid was obtained as the free acid.

The fractions containing the neutral and basic amino acids from the Dowex-1 column were combined and freed of acetic acid by evaporation *in vacuo*. They were then dissolved in a small volume of 1 *N* HCl and placed on a Dowex-50 (4% cross linkage) column which had been previously equilibrated with 1 *N* HCl. The amino acids were fractionally eluted by means of gradient elution using 1 to 4 *N* HCl. Alanine, glycine, and serine fractions were found by paper chromatography to be chromatographically pure. After the respective fractions were concentrated by evaporation *in vacuo* and evaporation to dryness several times to remove the excess HCl, the individual amino acid hydrochloride salts were converted to their free form in the following manner: The salt was dissolved in a small amount of distilled water and the free amino acid was absorbed on a Dowex-1 (OH^- form) column followed by washing the columns well with distilled water. The amino acid was then

eluted from the column with 0.5 *N* acetic acid and evaporated to dryness to recover the amino acid in solid form. Each amino acid was diluted to 3 millimoles with unlabelled amino acid and was crystallized from a water–alcohol mixture. For each amino acid, a small portion of the undiluted acid was reserved for the determination of its specific activity.

Degradation of Amino Acids

Aspartic and Glutamic Acids

Degradation methods used for these two amino acids were essentially those of Wang *et al.* (10, 11).

Alanine, Glycine, and Serine

Glycine was degraded by (1) combustion to CO_2 for the total activity of the entire molecule, (2) ninhydrin decarboxylation for the activity of the carboxyl carbon atom. The activity of the amino carbon atom was found by difference.

Alanine was degraded by total combustion to CO_2 for the specific activity of the whole molecule. Ninhydrin decarboxylation was used for the recovery of carboxyl carbon; the acetaldehyde formed was trapped in an ice-cold 1 *N* $K_2Cr_2O_7$–H_2SO_4 solution and immediately oxidized to acetic acid. The acetic acid was purified by steam distillation, isolated as sodium salt by titration with CO_2-free NaOH, and degraded according to the method of Phares (6).

The degradation of serine was carried out according to the method of Sakami (8).

Determination of Radioactivity

The radioactivity degradation products of amino acids was determined in the form of $BaCO_3$ mounted on cupped stainless steel planchets and counted by an end-window G–M counter. The samples were counted to $\pm 2\%$ standard deviation and corrected for background and self-absorption in the conventional manner.

C^{14}-Labelled Substrates

Glucose-2-C^{14} and glucose-6-C^{14} were furnished by the Bureau of Standards through the kind cooperation of Dr. H. S. Isbell.

Results and Discussion

The specific acitivities of the undiluted amino acids and the intramolecular distribution of C^{14} in each amino acid are given in Table I.

Alanine

This amino acid appears to be synthesized principally by transamination of pyruvate formed from glucose via the glycolytic (EMP) pathway. The specific activities of alanine from both substrates are equal (Table I), which indicates an equal contribution of pyruvate from both C_3 units of the glucose molecule. The minor C^{14} incorporation into C-1 and C-3 of alanine from glucose-2-C^{14}, and into C-1 and C-2 of alanine from glucose-6-C^{14} probably

TABLE I

DISTRIBUTION OF C^{14} IN SOME AMINO ACIDS DERIVED FROM LABELLED SUBSTRATES

Amino acid*		Distribution of isotope, %	
		Glucose-2-C^{14}	Glucose-6-C^{14}
Alanine	Total	1.62×10^5	1.62×10^5
	COOH	11%	5%
	$CHNH_2$	78	8
	CH_3	11	87
Glycine	Total	0.39×10^5	0.12×10^5
	COOH	17%	31%
	CH_2NH_2	83	69
Serine	Total	0.46×10^5	0.80×10^5
	COOH	7%	4%
	$CHNH_2$	41	6
	CH_2OH	52	90
Aspartic acid	Total	0.76×10^5	0.95×10^5
	COOH	23%	20%
	$CHNH_2$	32	35
	CH_2	21	42
	COOH	25	4
Glutamic acid	Total	2.35×10^5	2.91×10^5
	COOH	19%	8%
	$CHNH_2$	36†	22
	CH_2		32
	CH_2		35
	COOH	45	3

*Whole molecule in c.p.m./mM amino acid.
†Individual labelling in these carbon atoms could not be determined, owing to a laboratory accident.

reflects randomization through reversible CO_2 fixation reactions involving pyruvate and oxalacetate.

Serine and Glycine

Contrary to alanine, serine does not appear to be synthesized from pyruvate, but rather from glycine via C_2+C_1 condensation. The conclusion is drawn from the fact that the isotopic distribution patterns of serine are not compatible with those of alanine in the glucose-2-C^{14} experiment. As expected, there is a strong resemblance between relative C^{14} distribution in glycine and the corresponding portion in serine molecule (C-1 and C-2 of serine). Although the exact origin of the hydroxymethyl group in serine cannot be determined from the available data, it appears that C-2 of acetate, which is derived from C-6 or C-1 of glucose via the EMP–TCA pathway, was the ultimate major source for this group, the specific activity of which is relatively high.

It is noted that C-2 of glucose is incorporated into glycine much more than is C-6. This is understandable in view of the work of Weissbach and Horecker with spinach preparations (13), in which C-1 of ribose-5-phosphate was found to be incorporated into C-1 and C-2 of glycine to the extent of 10% and 80%

respectively. Presumably, the pentose formed by phosphogluconate breakdown undergoes a C_2–C_3 cleavage, giving rise to glyoxalic acid, which in turn serves as a precursor of glycine (1) (glucose C-3 = COOH; C-2 = amino carbon).

The pathway just described does not account for the incorporation of C-6 of glucose into glycine. The relatively small amount of C^{14} observed may reflect the presence of a minor pathway leading from methyl-labelled acetate, as derived from glucose via the EMP–TCA route, to the formation of glycine.

Aspartic Acid

The pattern (Table I) of biosynthesis of aspartate seems obscure on the basis of known reactions. The uneven incorporation of C-6 of glucose into this amino acid indicates that any four carbon precursor does not randomize extensively. Succinate or fumarate are thus ruled out as intermediates, and a C_3+C_1 condensation forming malate or oxalacetate from pyruvate (4, 14) also cannot account for these labelling patterns in view of the heavy incorporation of glucose C-2 into C-2 of alanine. However, inasmuch as glucose C-2 is incorporated fairly evenly into all positions of aspartate, it appears that the biosynthesis of aspartic acid in this organism may have involved smaller carbon fragments. Even here the number of possibilities is limited. Condensation of glyoxalate and acetate (15), for example, should on the basis of known reactions produce aspartate labelled largely as follows:

(3) (2) (1,6) (2,5)

$HOOC, CHNH_2, CH_2, COOH$. (Numbers refer to position of carbon atoms in glucose.) A $C_1+C_2+C_1$ condensation, which appeared possible when acetate was the sole carbon source (3), remains a possibility. However, the observed high incorporation from both C-2 and C-6 of glucose into C-1 or C-3 of aspartate and the low incorporation from C-6 of glucose into C-4 of aspartate render such speculation of limited value until further definitive experiments can be carried out, both with additional labelled substrates and with cell-free systems that may help to define the condensation reactions that transpire in this organism.

Glutamic Acid

Experimental findings reported previously (3) together with that given in Table I suggest strongly that this amino acid is synthesized from glucose intermediates via the conventional reactions in the TCA cycle. Thus, C-2 of glucose (corresponding to C-1 of acetate in the glycolytic pathway) was incorporated heavily into the carboxyl groups of glutamate, in nearly the anticipated 2:1 ratio (γ-COOH/α-COOH) (10). Similarly, C-6 of glucose, which corresponds to the methyl group of acetate in the EMP scheme, did not appear in great amounts in either of the two carboxyl groups of glutamic acid. The detection of a considerable amount of C^{14} in the middle carbon atoms of glutamic acid from glucose-2-C^{14} is in line with the observed labelling pattern of aspartic acid, which presumably represents the C^{14} distribution pattern of C_4 acid in the citrate formation step of the TCA cycle.

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INCORPORATION OF C^{14} INTO BACTERIAL PEPTIDES[1]

G. E. CONNELL[2] AND R. W. WATSON

Abstract

Alcoholic extracts of log-phase cells of *Pseudomonas hydrophila* grown on glucose as the sole carbon source were concentrated, extracted with ether, and dialyzed. After fractionation of the permeates by high voltage paper electrophoresis, five to seven ninhydrin-positive acidic, one neutral, and several basic bands appeared. As many as seven ninhydrin-positive basic bands have been found in some preparations, although they have been absent in others. Photometric analyses on eluates before and after acid hydrolysis indicated the presence of acidic, neutral, and basic peptides. Amino acid compositions of 28 of these peptides, separated by a combination of high voltage paper electrophoresis and paper chromatography, included all of the amino acids commonly found in proteins. Elution of acidic, neutral, and basic amino acid and peptide fractions from C^{14}-labelled cells was followed by measurement of total and specific radioactivities in carboxyl-C before and after acid hydrolysis. Total radioactivity was highest in proteins even 1 minute after addition of the isotope to a log-phase culture. Specific radioactivities, plotted against time, gave parallel hyperbolic curves with peptides below the curve for amino acids and with proteins still lower and almost linear. The evidence suggests rapid synthesis of peptides from amino acids.

Introduction

Peptides occurring in growing cells are of exceptional interest because of their possible bearing on problems of protein synthesis. Mere demonstration of peptides in an extract, however, is no proof of their presence in the original living cells. They may have been formed by proteolysis before or after the cells were collected, or they may have arisen as products of one or more side reactions during the extraction procedure. In many of the reports reviewed by Synge (32) of peptides isolated from cell and tissue extracts, precautions for inhibiting proteolysis in the freshly harvested cells, e.g. by cooling rapidly to the freezing point, were not taken. Consequently, there is no assurance that these peptides were not formed by proteolytic action or by some other synthetic or degradative process accompanying cellular disorganization. Many investigators have failed to accept these reports as evidence for the occurrence of metabolically active peptides. Campbell and Work (6) held that no significant quantity of any intracellular peptide other than glutathione had ever been isolated from a cell extract, although previous failures were attributed to lack of adequate technique. Recently, Borsook (3) recalled that repeated attempts to find intracellular peptides, even in very small amounts, using labelled amino acids, had failed.

Evidence has been presented by Turba and Esser (35, 36) and more recently by McManus (20) for the presence of metabolically active peptides in log-phase *Torula utilis* cells grown on acetate as the sole carbon source. Turba

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and Esser (36) suggested that these peptides might be formed: (*a*) as intermediates in protein synthesis, (*b*) by a side reaction of unknown function in the cell, or (*c*) by non-enzymatic polymerization of activated amino acids during the extraction procedure. In view of the obscure origin, structure, and function of the *Torula* peptides, other possible mechanisms for their formation come to mind. They may have arisen either in the living cell or during the extraction period through destruction of labile links in protein chains, or alternatively their accumulation in the cells may have been brought about through an impairment of protein synthesis effected by growth on acetate.

In experiments with C^{14}-labelled acetate Turba and Esser followed incorporation of the isotope into free amino acids, peptides, and proteins over short time intervals (36). In early stages amino acids and peptides became labelled more rapidly than proteins and the results were believed to be compatible with the view that intracellular peptides play a part in protein synthesis.

In the present investigation, in order to examine the possibility that intracellular peptides may be formed during the normal growth of a microorganism, alcoholic extracts of log-phase cells of *Pseudomonas hydrophila*, grown on unlabelled and also on C^{14}-labelled glucose as the sole carbon source, were analyzed for their peptide content. Preliminary reports on this work have already been published (8, 9).

Materials and Methods

Growth of the Organism

For the preparation of inocula *P. hydrophila* NRC 492 (33) was incubated 18 to 22 hours at 30° C on slants of dextrose agar containing 2% dextrose, 0.5% yeast extract, and 1.5% Bacto agar. Inoculum media, prepared by mixing four sterile stock solutions and containing 1% glucose, 0.6% $(NH_4)_2HPO_4$, 0.02% KH_2PO_4, and 0.025% $MgSO_4.7H_2O$, were allowed to stand before use to detect possible contaminants. Flasks remaining non-turbid were inoculated and incubated on a rotary shaker at 30° C for 20 hours. Purity of inocula was tested by plating and by the examination of Gram-stained smears.

For mass production of cells, 6 liters of culture medium, similar to the inoculum medium but containing 2% glucose, was prepared from the stock solutions. These solutions were added aseptically under ultraviolet irradiation to a specially designed 12-liter flask, fitted with sterile cotton air filters, air inlet, and outlet tubes and with a sampling device which allowed the withdrawal of uncontaminated 5-ml samples. Four such flasks were placed on a rotary shaker (110 r.p.m.) and aerated with 1 liter of sterile air per minute overnight at 30° C. If turbidity did not develop, 600 ml of 20-hour inoculum was added to each flask and incubation was continued. Samples were withdrawn at hourly intervals and optical densities (E660) were measured in a Coleman Junior spectrophotometer. A typical growth curve revealing the initial lag and prolonged log phase is shown in Fig. 1. Negative growth

acceleration commonly occurred after about 11 hours. After 9 hours, when the culture had reached an optical density of approximately 0.45, a final sample was removed for plating and for the preparation of smears. Growth was then suddenly terminated by pouring the contents of each flask, with vigorous stirring, into enough crushed ice to effect cooling to 0° C in less than 1 minute (36). Cells were separated by centrifugation at 50,000 r.p.m. (2°–6° C) in a Sharples supercentrifuge, and washed twice with distilled water

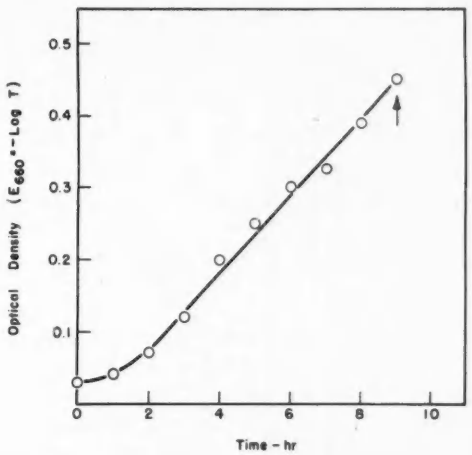

FIG. 1. Growth of *P. hydrophila* in 2% glucose + salts.

at 0° C in a refrigerated Servall SS-1 rotor at about 29,000 *g*. The average yield of wet cells was 5 to 6 g/l.; aliquots dried to constant weight approximated 12 to 13% dry weight. Packed in plastic 50-ml centrifuge tubes, the wet cells were rapidly frozen by immersion in liquid air and stored at −40° C.

Isotopically labelled cells were grown in a tower fermenter modified from the design described by Turba and Esser (36) and illustrated in Fig. 2. After sterilization the stopcock and standard joints were sealed with sterile grease and held by springs. The culture medium (1500 ml containing 2% unlabelled glucose) followed by 150 ml of a 20-hour inoculum was added aseptically through the side tube. Circulation of water through the jacket from a thermostatically controlled bath kept the culture at 30° C. Washed sterile air, forced at the rate of 1 liter per minute through the constricted tip of the central tube, effectively prevented sedimentation. For the rapid removal of samples the stopcock was provided with a 7-mm bore. Growth of cultures in the tower fermenter paralleled the curve shown in Fig. 1. Optical density readings versus cell numbers, estimated by plate counts (1), are plotted in Fig. 3. To label the cells with C^{14} the culture was allowed to grow for 8 hours on unlabelled glucose, until an anthrone analysis (22) showed about half the original glucose had been dissimilated. At this point, when the optical density

had reached 0.40 (cell count = 4.1×10^9/ml), 66 mg of uniformly labelled glucose with a specific activity of 10 μc/mg was added. Six samples of 250 ml each were withdrawn at intervals of 1, 5, 12, 30, 60, and 120 minutes after addition of the C^{14} glucose. During this 2-hour period the culture continued to grow logarithmically, reaching a final optical density of 0.51 (cell count = 5.0×10^9/ml). The cells were removed by centrifugation, washed, and stored at $-40°$ C.

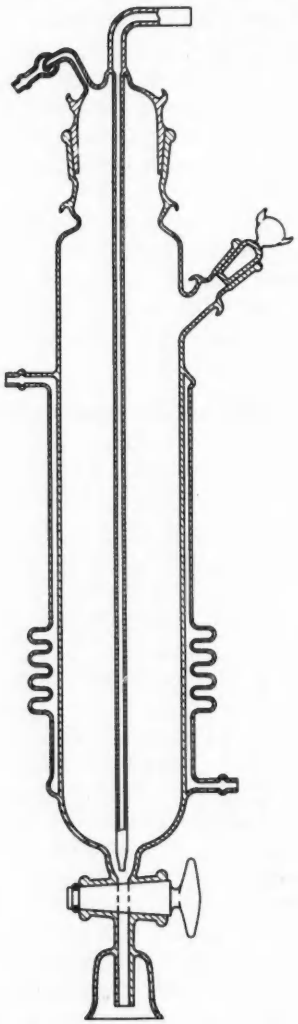

FIG. 2. Tower fermenter.

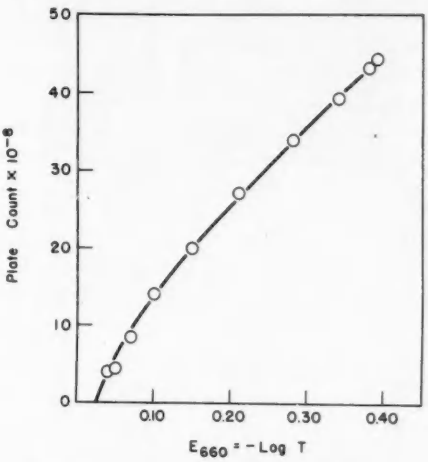

FIG. 3. Plate count vs. optical density.

Extraction of Peptides

Isolation of peptides for estimation of amino acid compositions began with the extraction of 10 g of frozen cells. With the temperature held below 0° C the cells were converted to a thin paste in cold 60% ethanol, pipetted into 100 ml of boiling 60% ethanol, and kept under reflux for 10 minutes. The supernatant liquid was removed after centrifugation at about 20,000 *g* and the residue re-extracted twice for 10 minutes each time with boiling 60% ethanol. To the pooled supernatant fractions 1.0 ml of 20% trichloroacetic acid was added to prevent foaming, the solution was concentrated *in vacuo* (12) to about 5 ml, diluted to 100 ml, and extracted three times with 50 ml ether. Concentration to dryness *in vacuo* over $CaCl_2$ left 280 mg of a glassy residue. Non-dialyzable material interfered with peptide separations by high

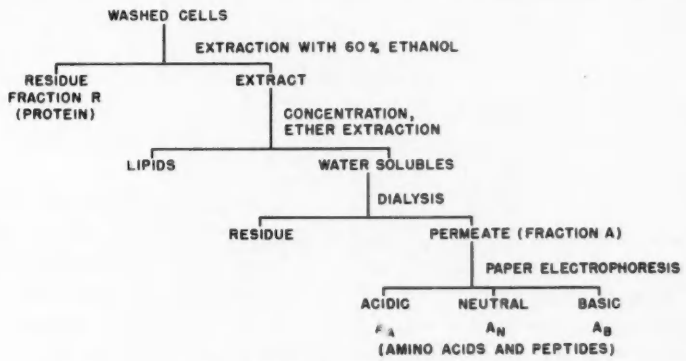

FIG. 4. Fractionation procedure.

voltage electrophoresis (HVE) so that the extract was dialyzed through Visking tubing for 24 hours against five changes of chloroform-saturated water. The total dialyzate recovered from the initial 10 g of wet cells weighed about 180 mg.

Peptides labelled with C^{14}, extracted in the same way from cells grown in the tower fermenter, were freed of alcohol-soluble protein by dialysis. The fractionation procedure is diagrammed in Fig. 4. Permeates were combined and reduced to dryness (fraction A). The residue (fraction R), after the third alcohol extraction, was dried and used for protein analysis. The non-dialyzable fraction could not be distinguished from the alcohol-insoluble protein on the basis of its specific radioactivity; since it contained less than 10% of the total protein, it was discarded.

Fractionation of Peptides by High Voltage Paper Electrophoresis

The entire dialyzate (180 mg) from 10 g of cells, taken up in 1 ml of water, was streaked with a micropipette in a narrow band on washed (7) SS2043B filter paper. Application of pyridine acetate buffer (16), at pH 6.5, in the manner described by Ryle *et al.* (29) was followed by electrophoresis for 45 minutes at 35 to 40 v/cm and 1.8 to 2.3 ma/cm breadth in the Michl apparatus (21). After removal and drying of the paper, bands were located by spraying edge strips with ninhydrin in chloroform (39), by the chlorine–starch iodide method (28) or by the Reindel-Hoppe procedure (25). Ninhydrin-positive bands obtained in this way are shown in Fig. 5. Bands were cut out and eluted with 25 ml of chloroform-saturated water containing 1% acetic acid. Eluates reduced to dryness were stored in a vacuum desiccator over Anhydrone.

Amino acids and peptides labelled with C^{14} were fractionated by HVE. Fraction A (Fig. 4) was dissolved in 0.10 ml of water and transferred quantitatively (allowing a small portion for radioautographs) to a strip of paper 11 cm broad. After 20 minutes at 35 v/cm the paper was dried and the position of the neutral band located by its fluorescence under ultraviolet irradiation. The eluate from this fraction was designated A_N, from the entire acidic fraction A_A, and from the basic zone A_B (Fig. 4). Elution was effected by extraction in a Soxhlet for 1 to 2 hours (15 changes) with 100 ml 40% ethanol, ethanol removed *in vacuo*, and the fractions stored in a desiccator. For radioautography 0.003 ml of the aqueous solution of each A fraction was transferred to filter paper for fractionation by HVE. A radioautograph obtained on Kodak no-screen X-ray film exposed for 18 days is shown in Fig. 6.

Estimation of Amino Acid Composition

Non-radioactive eluates from acidic bands (A_1 to A_7), from neutral (N) and from basic bands (B_1 to B_7), shown in Fig. 6, were further separated into component peptides and amino acids by repeated HVE and unidimensional paper chromatography in several different solvent systems: *n*-butanol–acetic acid (24), *sec*-butanol–formic–water (36), *n*-propanol–0.05 *M* sodium pyrophosphate + 2.1% NaCl (14), phenol buffered at pH 4 and 9 (19), and collidine–lutidine (2). To reduce the contamination with N-compounds from

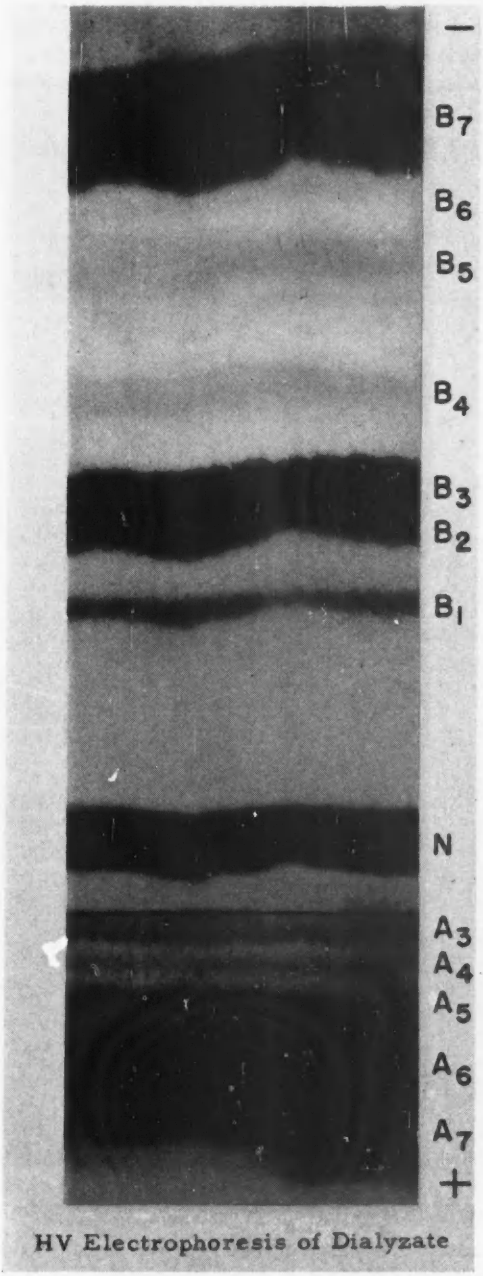

FIG. 5. Ninhydrin-positive bands obtained on HV electrophoresis of dialyzate: A, acidic; N, neutral; B, basic.

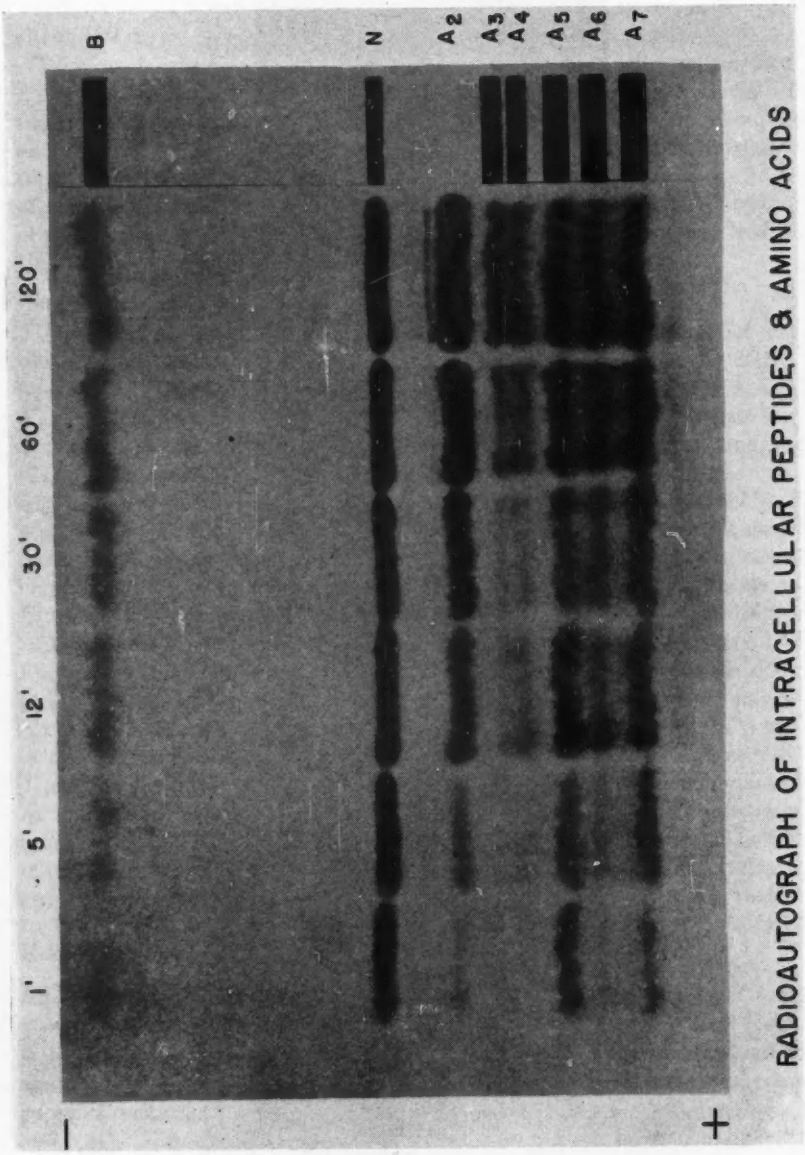

FIG. 6. Radioautograph of intracellular peptides and amino acids: A, acidic; N, neutral; B, basic. Black edge strips represent ninhydrin-positive bands.

washed filter paper, peptides were finally eluted from as small an area of paper as possible and paper blanks were compared. Eluates were concentrated to dryness, hydrolyzed for 16 hours in 6 *N* HCl, and constituent amino acids provisionally identified by paper chromatography.

Measurement of Radioactivity

Radioactivity in the carboxyl-C of free amino acids and peptides, corrected for background (160–170 counts per minute (c.p.m.)), were determined by the method of Sinex–Plazin *et al.* (31) on carbon dioxide released by the procedure of Van Slyke *et al.* (37) from eluates of fractions A_A, A_N, and A_B. Coincidence corrections were unnecessary since no fraction exceeded 50,000 c.p.m. Total activity calculations were based on entire 250-ml samples from the original culture: specific activities are expressed as c.p.m./mg carboxyl carbon. Peptide concentrations were calculated from differences between the radioactivities of hydrolyzed and unhydrolyzed portions.

Purification of Dialysis Tubing

Ninhydrin-positive compounds were present to the extent of 2 micromoles Ruhemann's purple per gram of Visking tubing. After hydrolysis the ninhydrin value of the extract doubled. Extraction with water in a Soxhlet for 12 to 15 hours did not appear to alter the properties of the tubing and removed 99% of the ninhydrin-positive compounds.

Preparation of Labelled Glucose

Glucose uniformly labelled with C^{14} was prepared by feeding $C^{14}O_2$ to photosynthesizing tobacco leaves and isolating the starch and pure glucose by the method of Vittorio (38).

Results

Amino acid compositions of 28 peptides are given in Table I. Acidic (A), neutral (N), and basic (B) bands are numbered from the origin. From the A_7 band, in which free aspartic acid was present, six peptides were isolated, each containing an unidentified ninhydrin-positive compound, X_1. An unidentified ninhydrin-positive band running ahead of A_7 was only occasionally present. From the A_6 band, which contained free glutamic acid, five peptides were separated. The neutral band contained nine free amino acids (α,ϵ-diaminopimelic acid (Dap), as well as Ala, Gly, Ileu, Leu, Ser, Thr, Tyr, Val (4)) along with one peptide containing 13 to 15 amino acids. Wide variations were noted in the composition of neutral peptides from different cultures. Dried permeates from one 10-g aliquot of wet cells were stored for a year in a vacuum desiccator over Anhydrone. At the end of this time 15 peptides were isolated from the neutral band alone, of which 7 were present in traces too small for investigation, and tentative amino acid compositions were obtained for the remaining 8 peptides (10).

Wide variation also occurred in the basic peptides from different extracts. From bands B_1 to B_7 in Fig. 5, eight basic peptides were isolated, as shown in Table I. Apparently no basic peptides were formed in the cells labelled with

TABLE I

AMINO ACID COMPOSITIONS OF PEPTIDES

HVE band	Amino acid composition	No. of amino acids	No. of peptides
B_8	Ala, ARg, Asp, Glu, His, Leu, Lys, Ser, Tyr, Val	10	1
B_7	Ala, ARG, ASP, Cys, Glu, Gly, Ileu, LYS, Met, SEr, Val	11	1
B_6	Ala, ARg, ASp, Cys, Glu, Gly, Leu, LYS, SEr, Tyr, Val	11	1
B_5	Ala, ARg, Asp, Cys, Glu, Gly, Leu, SER, Val	9	1
B_4	Ala, Arg, Asp, Glu, GLY, LYS, SER, Thr, Tyr	9	1
B_3	Ala, ARG, Asp, Glu, GLY, SER, Tyr	7	1
B_2–B_1	Ala, ARG, and/or LYS, Glu, and/or Tyr, Gly, Ileu, Leu, Met, and/or Val	7–10	2
N	Ala, Asp, Arg? Cys, DAP? Glu, Gly, His, Leu, Lys, Pro, Ser, Thr, Tyr, Val	13–15	1?
A_1	Ala, ASp, Glu, GLy, Leu, or Ileu, Lys, SER, Thr, Tyr, Val	10–11	1
A_2	Ala, ASp, Glu, GLy, Lys, SEr, Thr, Tyr, Val	9	1
A_3	ALa, Asp, Glu, GLY, Leu or Ileu, Lys, Thr, SEr, Val	9–10	1
A_4–A_5	1. Ala, Arg, ASP, Cys, Dap, GLu, GLY, Lys, Tyr	9	1
	2. Ala, ASP, Cys, Dap, GLU, GLY, Lys	7	1
	3. Ala, ASP, Cys, Dap, Glu, GLY, Lys, Try	8	1
	4. Ala, ASP, GLU, Lys, GLY, Leu, Try	7	1
	5. Ala, ASP, GLU, GLY, His, Leu, Lys, Met, and/or Val	8–9	1
A_6	1. Ala, Asp, Dap, Glu, GLY, Ileu, Met, SER, Thr, Tyr, Val	11	1
	2. ALa, ASp, Dap, Leu, Lys, Phe, SEr, Thr, Val	9	1
	3. Ala, ASp, Dap, Glu, GLY, Leu, Lys, Phe, PRo, Ser, Tyr	11	1
	4. Ala, Asp, CYS, DAp, GLU, GLY, Lys, Pro	8	1
	5. Ala, Asp, Glu, Gly, His, Ser, Thr	7	1
A_7	1. Ala, Asp, Dap, GLU, Gly, Xi	5	1
	2. ALA, GLU, GLY, Leu, Lys, SER, VAL, Xi	7	1
	3. ALA, ASP, Cys, DAP, GLU, Gly, Val, Xi	7	1
	4. ALA, ASP, Cys, GLY, Lys, SER, THR, Val, Xi	8	1
	5. ALA, ASP, GLU, GLY, Leu, Lys, SER, Tyr, Val, Xi	9	1
	6. ALA, ASP, Cys, GLU, GLY, Leu, Lys, SER, Tyr, Val, Xi	10	1
			28

NOTE: Bands are numbered from the origin: The relative concentrations of the amino acids are indicated by the number of capital letters in the abbreviations (4): A, acidic; N, neutral; B, basic.

C^{14}. The single ninhydrin-positive band marked B in Fig. 6 was identified chromatographically as histidine. This absence of basic radioactive peptides was confirmed by the finding that no measurable increase in $C^{14}O_2$ released by ninhydrin (37) occurred after hydrolysis of fraction A_B. The total number of peptides isolated from *P. hydrophila* now stands at 43, of which amino acid compositions have been obtained for 36. These represent minimum estimates, since other peptides were present in amounts too small for analysis, since they were ninhydrin-negative, or since, for other reasons, they escaped detection.

Relative concentrations of peptides, calculated from increases in ninhydrin color after hydrolysis by the Troll–Cannan procedure (34), are given in Table II. Two- to four-fold increases occur in the acidic and basic zones, with only a slight increase in the neutral eluate. Calibrated on a standard leucine curve, the total as leucine released on hydrolysis is 6.4 mg per 10 g of wet cells. Dry weight approximates 12.5% of the packed cells, so that ninhydrin-positive

TABLE II

INCREASE IN NINHYDRIN COLOR OF PEPTIDE BAND ELUATES AFTER HYDROLYSIS

Band No.*	Concentration in eluate		Total (as leucine) released on hydrolysis, μg	Increase in ninhydrin color after hydrolysis, %
	Before hydrolysis, μg	After hydrolysis, μg		
N	2610	3000	390	15
B_1–B_2	450	875	425	94
B_3–B_4	375	875	500	75
B_5–B_8	450	1375	925	203
A_1–A_3	250	875	625	250
A_4–A_5	350	1500	1150	328
A_6	325	1500	1175	362
A_7	1375	2610	1235	90

*Band numbers refer to Fig. 5. Concentrations are expressed as micrograms of leucine from Troll–Cannan procedure (34) using standard leucine curve.

compounds in combined form may be present to the extent of 0.5% of the dry weight. Deammonification and subtraction of paper blanks would lower this value.

Rapid labelling of amino acids and peptides in log-phase cells is revealed by radioautography in Fig. 6. At least three of the five bands migrating toward the anode contain ninhydrin-positive compounds which are not amino acids. Although di- and tri-carboxylic acids may account for part of the radioactivity in this area, part at least must be due to peptides. Band A_2 corresponds to a yellow pigment. From the increase in radioactivity of $C^{14}O_2$ released after hydrolysis, the neutral band N presumably contains one or more radioactive peptides. No radioactive peptides migrated toward the cathode.

Incorporation of the isotope into amino acids and peptides, measured as total radioactivity in the alcohol extracts from 1 to 120 minutes after addition of C^{14} glucose to the log-phase culture, is shown in Fig. 7. Activities in the carboxyl groups of free amino acids are represented by the lower curve. The

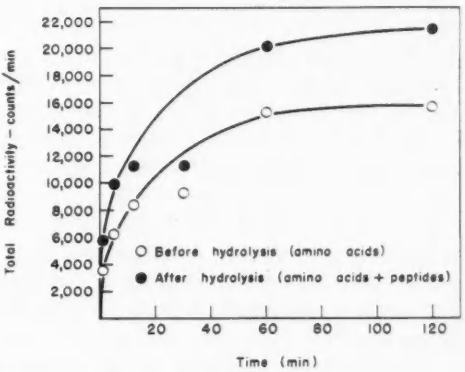

FIG. 7. Total radioactivity in alcohol extracts vs. time.

upper curve gives measurements after hydrolysis, so that activities of carboxyl groups in peptide linkages may be obtained by difference. Of the free amino acids, the neutral fraction contributed 65% of the radioactivity, the acidic fraction 25%, and the basic fraction 10%. Neutral peptides contribute 40 to 50% of the activity in peptides and acidic peptides the balance.

The combined activity of amino acid and peptide fractions is compared with the total activity in proteins in Fig. 8. In contrast to the results of Turba and Esser (36) protein activity exceeds that of amino acids and peptides even at short time intervals. Considerable differences are suggested between concentrations of amino acids and peptides in cells of *P. hydrophila* and *T. utilis*. From our radioactivity measurements the approximate concentrations per gram dry weight of cells were: amino acids, 6 mg; peptides, 3 mg; alcohol-insoluble protein, 400 mg.

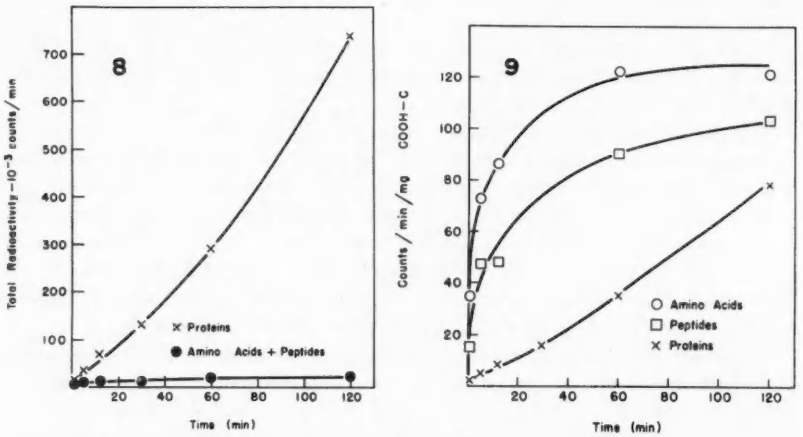

FIG. 8. Total radioactivity in amino acids plus peptides and in proteins.
FIG. 9. Specific radioactivities in amino acids, peptides, and proteins vs. time.

From the specific activities presented in Fig. 9 amino acid and peptide curves follow a parallel course, rising sharply from the origin and levelling off after approximately 90 minutes. Specific activities of neutral and acidic peptide fractions were identical within experimental error: that of neutral amino acids increased slightly faster than that of acidic and basic amino acids.

Discussion

The main questions, which arise from this preliminary study, concern the extra- or intra-cellular locus and the mechanism of formation of the peptides. In the following discussion evidence with a direct or an indirect bearing on these questions is assessed.

Are the peptides present as components of the growing cells? The extremely low rates of enzymic reactions in cells at 0° C (36) suggest that protein

degradation in the 2-hour period from the time of harvest of the cells to final storage at −40° C cannot account for peptide formation. Extracts of comparable microorganisms, prepared in the same way, reveal no trace of peptides, using procedures that would be favorable for the detection of small peptides (5, 11). Neither do variations in the extraction procedure seem to change the concentrations of peptides present in the extracts. Substitution of boiling water for boiling alcohol, and addition of cold alcohol to a sonic extract of the cells yield similar patterns of acidic peptide bands (18). If the peptides are formed by random non-enzymatic polymerization of activated amino acids during the extraction procedure, the pattern should vary from one sampling time to another. Instead, the same distribution of peptides in bands is preserved through six sampling periods varying from 1 minute to 2 hours (Fig. 6). Since peptide bonds in proteins are not known to be split in boiling alcohol or in boiling water, the evidence at hand provides an indication that the peptides are not formed either by non-enzymatic polymerization of activated amino acids, nor by proteolysis during the extraction period. The appearance of peptides in the extracts suggests that they are present in the cells during log-phase growth. Experiments designed to answer this question are under examination.

Are peptides formed in the cells by proteolysis? The results of the present investigation do not exclude the possibility that the *P. hydrophila* peptides may be formed within the cells by degradation of protein. In Fig. 8 the protein curve does not pass through the origin, and the total radioactivity of the proteins is greater than that of amino acids plus peptides even in the 1-minute sample. Although this observation may mean that the proteins were not completely freed of adsorbed peptides and amino acids, it may alternatively be due to the presence of one or more rapidly formed labile intermediate proteins with a high turnover rate. If we assume for the purpose of argument that non-radioactive peptides are present in the cells at the moment of addition of the isotope, proportionally so many more non-labelled proteins are present that their specific radioactivities, after short time intervals, are not strictly comparable to those of the low-molecular compounds. If newly formed protein, synthesized after the addition of C^{14} glucose, could be separated, and its isotopic content measured, its specific activity could conceivably be higher than that found for peptides. The possibility that the labelled peptides may be formed by the degradation of newly formed protein of high specific activity cannot, therefore, be excluded on the present evidence. By analogy, however, the explanation appears unlikely. Within recent years the concept of the "dynamic state" of proteins in exponentially growing microorganisms has been challenged. Hogness, Cohn, and Monod (15), Rotman and Spiegelman (27), and Koch and Levy (17) have provided evidence that protein turnover in log-phase cells of *Escherichia coli* takes place at a scarcely measurable rate if it occurs at all. Halvorson (13) has reached a similar conclusion from his work on *Saccharomyces cerevisiae*. If it is also true

that protein turnover is immeasurably slow or non-existent in log-phase cells of *P. hydrophila*, the peptides reported in the present investigation are probably formed directly from amino acids.

There are both similarities and differences between the peptides from *P. hydrophila* and those reported by Turba and Esser from *T. utilis* (36). In both organisms the peptides become labelled rapidly when C^{14} is provided in the carbon source. With 43 peptides now isolated from *P. hydrophila* and 42 peptides reported from *T. utilis*, the numbers present in the two species are in the same range. Although *P. hydrophila* produces concentrations of peptides during log-phase growth on acetate (10), xylose (23), and glucose, it is possible that *T. utilis* peptides may be formed through an impairment of protein synthesis. Considerable efforts were made by Cowie and Walton (11) to find peptide linkages among the amino acids in cold trichloroacetic-acid-soluble fractions of *T. utilis* grown on fructose as the sole carbon source and no peptides were found. Neither were peptide fragments observed as transient intermediates in *E. coli* grown on glucose, salts, and ammonia by Britten, Roberts, and French (5). The data of Cowie and Walton carry the suggestion that the appearance of peptides in extracts of *T. utilis* is correlated with growth of the cells on acetate.

Other significant differences between the two species were also observed. In *P. hydrophila* labelling in acidic, neutral, and basic fractions occurs within 1 minute of addition of the isotope, with the greatest incorporation occurring in neutral compounds (Fig. 6). By contrast, labelling of acidic compounds occurred in the earliest stages in *Torula*, with neutral compounds becoming faintly radioactive only after several minutes. Aspartic acid, a common component of the peptides of *P. hydrophila*, is of rarer occurrence in the *Torula* peptides; basic compounds from *Torula* were in low concentration or absent. These differences probably reflect different metabolic patterns in the two species on the different substrates. Acetate carbon would be incorporated more rapidly in proportion into glutamic acid and its derivatives (26). Rapid incorporation of the isotope into the protein fraction of *P. hydrophila* in relation to amino acids plus peptides is correlated with the accelerated rate of protein synthesis in the bacterium. Total radioactivity of the amino acids and peptides of *P. hydrophila* continued to increase during the course of the experiment (Fig. 8), while in *T. utilis* it declined after 60 minutes. This decline in the yeast may be correlated with a decreased rate of growth and concomitant reduction in the concentration of amino acids and peptides. Since α,ϵ-diaminopimelic acid (Dap) appears to be a component of about one third of the peptides from *P. hydrophila* (Table I), peptide formation may be correlated with the synthesis of cell wall materials (30).

Acknowledgment

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CORRECTION

Volume 4, pages 174, 175. The illustration that appears opposite p. 174 is Plate II, not Plate I as labelled, and should face p. 175. The illustration facing p. 175 is Plate I, not Plate II, and should face p. 174. The captions are in correct position.

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